A Platform Technology for Optimized Production of Islet-like Spheroids and Other 3D Cell Spheroids for Applications in Drug Discovery and Regenerative Medicine

By

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ABSTRACT

There is an ever-growing need for a transition from the flat world of 2D monolayer cell culture to one of 3D organotypic tissue structures, bringing increased physiological relevance to early bench-top scientific discoveries. The development of simple methods for 3D cell culture is essential to gain acceptance among researchers and industry. This dissertation presents a novel glass micromold that allows optimal engineering of cells into a 3D cell spheroid. When cells are loaded onto the micromold, they settle into micro-wells, conical-shaped recesses, and are cultured for several days until a cell aggregate has formed. In diabetes, islets are dispersed into single cells, loaded onto the micromold, and engineered into islet-like spheroids called Kanslets. Native islets exist in a broad size range (50 to 400 μm in diameter) and inherently contain various size-dependent limitations. To overcome these barriers, Kanslets are limited to a uniform size range (diameter < 125 μm) during cell reaggregation. While Kanslets are organotypic spheroids that mimic the structure and function of native islets, they have a superior viability and a decreased diffusion barrier. These qualities are essential for drug testing and islet transplantation, where diffusion is the primary mode of nutrient transport. Diabetic rats that received Kanslet transplants regained insulin independence similar to those receiving native islet transplants. For the high-throughput drug screening, size-optimized Kanslets were easily dispensed and seemed to respond to known drugs in the appropriate fashion. These micromolds were also used to produce cancer cell and stem cell spheroids, to demonstrate the wide applicability and use for a variety of fields. This dissertation sets the

foundation for developing and leveraging a technology that aims to bridge the gap between *in vitro* experimentation and future clinical use.

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CHAPTER 1: Introduction to Dissertation

What began as a project to engineer optimal islet tissue for transplantation for the treatment of type 1 diabetes, transformed into the invention of a platform technology for producing 3D cell clusters. The innovations within this technology enable the production of various 3D cell spheroids with applications in drug discovery, regenerative medicine, and a variety of other fields. This dissertation focuses on using this technology in diabetes and provides an exploration into various future applications.

The overall objective of this dissertation was to engineer optimal islets using micromolds for transplantation for type 1 diabetes. The specific aims of this research were to: (1) develop a micromold to engineer islets from dispersed, single cells, (2) develop suitable aggregate tissue culture conditions for reaggregation and natural islet function, and (3) characterize the diffusion properties and function of engineered rat islets *in vitro*.

In accomplishing these goals, I developed a device on which dispersed islet cells could be optimally reaggregated into 3D islet-like cell clusters. I then examined whether these engineered islets provide a physiologically relevant model for diabetes that overcame several of the barriers that hamper the use of islets in the clinic. In addition to the original goal, I explored the basic application of this technology as a platform in various fields for future development.

Chapter 2 provides a detailed background of pharmaceutical drug development and the current methods used to find new molecular entities, with a discussion about their advantages and disadvantages. In focusing on diabetes, we

identify a necessity for more suitable technologies in the diabetes drug discovery market.

Chapter 3 provides support for the necessity to engineer islet cell clusters and presents the development of a novel technology for 3D cell culture. This novel technology begins with the development and fabrication of a micromold device for the optimal reaggregation of dispersed, single islet cells into 3D islet-like cell clusters, or Kanslets. I examine the feasibility of the technology for commercial use and high throughput drug screening.

In chapter 4, Kanslets, one of the resulting 3D cell structures, are characterized and compared to isolated, native islets. Cell viability, glucose diffusion and uptake, composition, morphology, and function are among the various factors characterized. A comparison to native islets provides the rational for utilization of Kanslets as an alternative for drug discovery and transplantation. For its application in regenerative medicine, preliminary Kanslet transplantation results provide support as a proposed treatment for diabetes.

Chapter 5 expands on the use of micromold technology as a platform for the development of 3D tissue spheroids using different cell types including cancer and stem cells, in applications including drug discovery and regenerative medicine. The need for technology in these various fields is combined with our preliminary data to provide support into the future of this platform technology.

The dissertation concludes in chapter 6 with a discussion about the next stages of research and development necessary to progress this technology for future application in various markets.

CHAPTER 2: An Examination of Current Technology for Drug Discovery and its Implications for Next Generation Therapies for Diabetes.*

ABSTRACT

Researchers are increasingly embracing the transition from the traditional, flat world of 2D cell culture to the organotypic nature of 3D tissue culture. These 3D tissue spheroids provide a more physiologically relevant model for studying various diseases. Cancer researchers have already begun the change to *in vitro* tumor spheroids as a better model. This is not the case for diabetes drug discovery and research. Beta-cell monolayers are currently in use for their ability to simulate the insulin-producing beta-cells within the pancreas. Unfortunately, these transformed cell lines are a less than ideal model to mimic the multi cell type islet. In this review we examine the current methods for drug discovery and high throughput screening, and discuss its relationship in discovering the next generation of therapeutics to treat diabetes.

INTRODUCTION

Pharmaceutical drug development is generally an expensive venture costing in excess of a billion dollars for each drug that is approved for market use. As the pharmaceutical industry looks toward discovering new drugs for market, there is an ever-growing need to increase the efficiency of screening large libraries of chemical

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compounds with the high probability of finding the most valuable and successful hits. As innovative technologies and techniques are being developed, the recent trend in drug discovery and high-throughput screening (HTS) is a paradigm shift from two-dimensional (2D) to three-dimensional (3D) cell culture. Emphasis is being placed on more physiologically relevant models for use in drug discovery and development. This relevance comes through transition from a traditional 2D monolayer cell culture, to a multicellular, organotypic 3D cell structure. The pharmaceutical industry primarily utilizes 2D assay technologies that tend to be less accurate and often times do not predict *in vivo* outcomes. Ultimately leading to longer drug development timelines, increased failure rates, and increased costs. The utilization of 3D cell cultures offers a model that better mimics the natural *in vivo* structure and function of native tissues, yielding stronger correlations between *in vitro*, cell-based experimentation and subsequent *in vivo* responses. Transitioning to this approach provides a more suitable solution for the discovery and development behind the next generation of drugs and therapeutics.

COST OF BRINGING NEW MEDICINAL ENTITIES TO MARKET

The process of discovering novel drugs and bringing them to market is quite complex (Figure 2.1), but it can be consolidated into 3 major stages: discovery, preclinical, and clinical [1]. A discovery phase generally involves target identification and validation, assay development, screening, and lead identification. Once suitable drug compounds (leads) are identified, they are subject to extensive *in vitro* testing (lead optimization) and animal model testing during the preclinical

stage. After enough supporting data has been gathered, the drug candidate transitions from animal into human trials during the clinical stage.

The discovery phase is essential to the success of lead compounds that move onto preclinical trials. Target identification, validation, and assay development are at the foundation of successful hit to lead generation. Several years of research and development (R&D) are placed behind these assays, as they are critical in finding high quality hits. Further assaying and discovery narrows the list of hits to a key selection of high quality lead compounds. More money and time is invested in optimizing these leads and developing them into the pharmaceuticals that will enter preclinical and clinical trials. The complexities and intricacies involved with each stage can easily generate problems that increase development time and costs, and may even lead to failure and removal from the market.

Despite spending of billions of dollars by industry per year on pharmaceutical R&D, only a handful of new medicinal entities (NME) are approved through the regulatory process [2]. In 2010, biopharmaceutical companies invested nearly $67.4 billion on R&D in find new medicines [3]. Recent estimates suggest that it costs an average of $1.8 billion per NME that makes it to market [4]. Much of this cost is due to the high attrition of NMEs that fail during development, particularly during later stages including Phase II and Phase III clinical trials, accounting for roughly 75% of the total costs [4-10]. According to a recent report, in 2011 alone, 31 drugs failed during late-stage clinical trials, with over half of the failures attributed to lack of efficacy [11]. The R&D timeline for most pharmaceuticals range from 10 to 15 years, with the success rate of reaching market at a mere 11% [4,6,7].

The 20-year life span of a utility patent from initial application means that a long development time leaves little room to generate revenue—let alone, enough revenue to recover the cost of R&D. Although resources and effort are being put into decreasing the development timeline to provide more patent exclusivity and gather revenues post-development, there is still much room for improvement.

DRUG DISCOVERY

A crucial factor that would increase success rates is the appropriate selection of NMEs before they enter further development. There are several methods of discovery including molecules from natural products, repositioning of drugs on the market, rational drug design, and combinatorial chemistry. If the initial candidates chosen for continued testing had a higher predictability and accuracy, the success rates of NMEs to market could significantly increase. This would prevent potential "positive hits" from undergoing extensive development and testing before they are ultimately deemed as failures.

Natural Drug Products

There are several methods utilized for drug discovery and lead identification. One of the oldest and most successful methods to find new drugs has been the utilization of natural products or natural product-derived compounds, and continues to play a major role as a novel lead generator [12-17]. Natural products are found in plants, insects, reptiles, marine life, microogranisms, and a number of other sources. These compounds have produced some of the world's most well

known drugs including aspirin, caffeine, penicillin, tetracycline, paclitaxel—and the list continues [13,14,18]. Paclitaxel, sold by Bristol-Myers Squibb as Taxol, a compound from the bark of the Pacific yew tree, *Taxus brevifolia*, was discovered to have anticancer activity and is currently used as a chemotherapy against lung, ovarian, breast, head and neck cancers [19]. Natural sources are highly sought-after for their diverse chemical nature. Access to unique sources can limit discovery, while supply and plant or animal extinction can further hamper efforts.

Drug Repositioning

A low risk and cost-effective approach to drug discovery is the repositioning of existing drugs and therapies into new markets or for new indications. Repositioning allows for a quicker time to market with reduced risks [2,20,21]. There are several ways that drug repositioning can occur. As patients use drugs available on the market, physicians and scientists continue to gather data, sometimes providing support for a new indication as opposed to its original intended use. Additionally, during clinical trials, it may be found that a drug is not effective for its intended target indication, but shows benefit for a different indication. Drugs repositioned during clinical trials come with support from the extensive amounts of gathered data about efficacy, pharmacokinetics, toxicology, and safety. Repositioning the drug before it is considered a failure can be advantageous, as long as no major concerns including safety were raised during the clinical trials.

The regulatory pathway for repositioning drugs already on the market is less risky and requires far fewer resources, further decreasing costs and time to market. Filing additional method-of-use patents is often required to solidify commercialization efforts, but can be legally hard to navigate as compared to *de novo* (new) drug discovery [21]. Dapoxetine was successfully repositioned in 2001 as a treatment for premature ejaculation after failed attempts as an SSRI (selective serotonin-reuptake inhibitor) to treat depression [21].

Rational Drug Design

Rational drug design was initially hailed as the answer to the challenges associated with drug discovery. This approach involves the creation of a small molecule to act on specific biological target based on the current knowledge of the target and desired therapeutic effects. The basic idea is to construct an appropriate ligand (small molecule) that will bind to a target to produce a "drug-like" effect. This approach to drug development is often times computer-assisted and involves knowledge of the 3D structure and function at the biomolecular level of the target, known as structure guided-computer aided drug design [22]. Although this can be used in designing NMEs, it tends to be employed during drug development and designing chosen drug candidates for better efficacy or to produce a more specific result [23].

Combinatorial Chemistry

To date, the Food and Drug Administration (FDA) has only approved one compound for clinical use that was discovered through combinatorial chemistry, while many remain in various stages of development. In 2005, Sorafenib was approved for treating renal cell carcinoma and became the first approved drug discovered through a combination of combinatorial chemistry and high-throughput drug screening [24]. Combinatorial chemistry creates a large library of diverse but structurally similar compounds—providing variations on molecules or compounds known to produce "drug-like" activity. When these combinatorial libraries are used for discovery, it increases the probability of finding new and unique compounds, and can be equated to finding a needle in a haystack [17]. These active compounds may provide increased efficacy, decreased toxicity, or provide a different mechanism of action in comparison to an original drug-like molecule. Combinatorial chemistry provides another method of rational design in developing NMEs.

In general, drug discovery aims to identify the candidate molecules that produce a therapeutic effect. In all these methods of discovery, there is one common goal: finding NMEs that will successfully become marketable pharmaceutical drugs. More importantly, this goal must be completed in a timely and cost efficient manner. This necessitates selecting the most likely to succeed drug candidates to move through the R&D pipeline. Compound selection plays a key role in success, especially considering it sets the precedence for the next decade or

more of time and resources. Failure to work on the best candidate from the beginning increases the cost to produce marketable NMEs exponentially [5,7].

HIGH-THROUGHPUT SCREENING (HTS)

In identifying lead compounds on which to focus R&D efforts, the pharmaceutical industry is increasingly utilizing high-throughput screening (HTS). HTS allows screening of large libraries of chemical compounds to search for those that have an effect on a target. Over the last few decades, HTS capacities have increased from manual screening methods consisting of several hundred screens per week, to highly automated screens processing roughly 100,000 samples per day [17]. A high-throughput screen is typically performed with 100,000 individual trials occurring in 96-, 384-, or 1536- well plate formats using automated machinery for both dispensing and assaying. Emerging technologies present future opportunities to process over 100,000 samples per day and are being termed ultra-high-throughput screens (uHTS). Many automated HTS systems are run 24 hours a day, 7 days a week, to offset the costs of ownership and maintenance. This combination of automation, high-density format, and high output of data play a significant role in discovery cost reduction. In fact, over the last 2 decades, the advancement of HTS technology has lead to significant timesaving during discovery and a 10-fold reduction in testing costs [25,26]. Few argue that even with these benefits and the significant capital that has been invested in HTS to date, it has achieved minimal success [21,26,27].

The basic foundation of HTS relies on a large compound library. These libraries are built though the utilization of one or more of the previously discussed methods of drug discovery. In combination with HTS, sampling thousands of compounds against a target significantly reduces the time required to find lead compounds. A typical screen may consist of 100,000 samples and yield between 100 to 500 primary hits [17]. Compound libraries, are the core of HTS, consisting of several thousand to over a million individual compounds. These libraries are created using one or a combination of drug discovery methods. Although the primary screen may illuminate which hits represent "drug-like" activity, subsequent secondary studies are conducted to further verify these findings before choosing the most appropriate lead candidates. Despite acceptable success rates for compounds found during HTS moving on to lead optimization, the continued high rate of false-positives demands the development of more accurate and reliable screening technologies [28].

In conducting a high-throughput screen, there are two broad categories of assays to choose from: biochemical assays or cell-based assays. Developed and highly utilized in the 1990s, biochemical assays provide a direct approach in determining compound activity on an isolated target of interest [29,30]. This assay provides target-based assessment of biochemical interactions such as enzymatic activity, receptor-ligand binding, or protein-protein interaction [29]. Although *in vitro* biochemical assays help elucidate potential drug activity, isolated targets are tested in an artificial environment. Biological systems are much more complex with many redundancies for protection, and this can alter a drug's *in vivo* effect.

CELL-BASED ASSAYS FOR HIGH THROUGHPUT SCREENING

To warrant the capital investment in HTS technology, emphasis must be placed in using methods that will provide the highest success rate. The introduction of cell-based assays accommodates for observing the effects of compounds directly on biological systems. The current use of 2D cell-based HTS technologies as a primary tool for screening large compound libraries aims to produce higher quality data [31-33]. Compounds are tested on cells to account for complex biological processes. Many of these technologies have a proven track record for a quick and low-cost method to discover potential drug candidates, with many discoveries currently in the development pipeline. In most cases, the direct target on which the test compounds act upon is unknown; instead hits occur due to complex cellular pathways [29]. In a primary screening, a well-established cell line is dispensed, using automated equipment, in a flat (2D) monolayer on the bottoms of 96-, 384-, or 1536-well plates. Cells are subject to compounds and assayed for effects including metabolism, proliferation, cytotoxicity, or function. After a list of potential compounds is narrowed, a secondary screen is conducted to further determine the most viable leads.

There are many advantages to cell-based assay technologies compared to biochemical assays. A cell-based assay provides a more physiologically relevant platform enabling living cells to be assayed after direct exposure to drug compounds. In this environment, it is possible to discover NMEs for a variety of pharmacological effects. The complexities of intercellular activity allow the discovery for either agonists or antagonists [34]. Drugs can act along various

chemical pathways within a cell, leading to a true phenotypic screening approach. The researcher knows the end effect measured in the cell, but may have no concept of the mechanism of action with the primary screen. They may set off a cascade of responses within a cell that may or may not have a beneficial effect, and allows identification early in the discovery process. This is akin to how medicinal discoveries were made before modern science, by noting the effects of natural substances on human beings. Employing cell-based assays provides more accurate, tissue-specific results that can improve reliability and translation between all stages of drug development [33,35].

Although 2D cell-based assays provide a simplified primary screening tool, they lack several key essentials necessary to accurately and predictably determine the best candidates[36]. A large number of false positives and false negatives can be mixed among the vast amount of data and requires scrutinizing true hits versus artifacts [34]. Additionally, compounds that show little or no effect during HTS may truly be potentially beneficial drugs if they were moved forward into clinical trials. During lead optimization, several cell-based assays are conducted concurrently to further characterize and develop features such as efficacy, potency, safety, and selectivity [34]. Lead optimization is an essential step in the pathway of drug development success. Studies using an unsuitable lead means time, money and resources may be spent on products that end up failing. As mentioned previously, a large amount of drug failures occur during the preclinical stage, especially during functional cellular and animal studies. In the current approaches to drug discovery, several inherent problems such as this may play a contributing factor in the longer

development times, greater expenditure, and high attrition rates that plague drug development.

An examination of the drug discovery process demonstrates various inconsistencies that can be traced back to the limitations of using 2D cultured cell monolayers. To start, primary cells (wild type, unadulterated, non-transformed cells taken directly from the source) have a limited ability to proliferate in culture, de-differentiate over time, and result in altered phenotype during passaging [35,37]. Unlike cancerous cells, when primary cell types are explanted and cultured in an *in vitro* environment, they must often be genetically manipulated to support proliferation and maintain a differentiated state. This cancer-like transformation enables stable cell expansion through passaging, creating an immortalized cell line. Immortalized cell lines can be proliferated indefinitely without changes to their functionality as compared to primary cells. Cell transformation can have advantages including revealing the effects of genetic manipulation and the reproducibility of a specific batch of cells. Unfortunately, these genetic modifications change cell phenotype over time, producing new abnormalities, and lead to alterations or loss of native functions and responses, ultimately affecting *in vitro* testing [37].

To complicate the situation, cultured cell lines are traditionally grown as a flat, monolayer of cells adhered to a cell culture surface. Tools for cell culture are available with modified surfaces to enable monolayer growth and proliferation, but often times cell lines are further modified or transformed. Now, in addition to the altered phenotype, the mere fact that the cells are in a 2D state affects study outcomes. In the last decade, extensive amounts of research have shown that cells

cultured in a 2D environment lack efficacy in predicting *in vivo* responses in a 3D environment and may exhibit unnatural characteristics [38-48]. Within our body, cells exist in a complex three-dimensional environment that provides support for cell-to-cell communication, mechanical stimulation, and biochemical signaling, among a number of factors that play a critical role at the cellular, tissue, and even organ level.

The failure of 2D monolayer culture to predict future efficacy is well documented in cancer research [38,41,49-51]. In fact, a recent study by researchers at the University of Kansas Medical Center found that 70 to 80% of the drugs they tested *in vitro* on 3D tumor spheroids showed varied results when compared to 2D monolayers of the same cell type [49]. Preclinical animal studies are the current avenue to provide the best prediction of *in vivo* responses to NMEs. Shifting paradigms from 2D to 3D cell culture, will not only provide a more relevant model for *in vivo* responses earlier in the drug discovery process, but may inevitably reduce the number of animals studies necessary to bring a drug to market. Combine this with maximizing the probability that elected lead compounds are successful and you effectively decrease drug development costs and attrition rates.

DRUG DISCOVERY AND DIABETES

Diabetes mellitus, commonly referred to as diabetes, is the 7th leading cause of death in the United States—affecting 8.3% of the population, equating to over 25 million individuals [52]. The Centers for Disease Control predicts that by 2050, 1 in 3 to 1 in 5 Americans will have diabetes (whether diagnosed or undiagnosed) [53].

Worldwide, roughly 350 million individuals are affected and the International Diabetes Federation estimates its prevalence to reach 1 in 10 by 2030 [54]. The United States faces an economic burden with expenditure and lost productivity totaling $174 billion [55]. Diagnosed individuals spend on average $11,744 annually on healthcare [55].

The hallmark of diabetes is the body's inability to produce or utilize insulin, leading to an abnormal maintenance of blood glucose levels. In type 1 (insulin-dependent) diabetes, autoimmune destruction of the beta-cells halt production of insulin. Type 2 (insulin-resistant) diabetes is caused by the body's inability to properly utilize insulin and accounts of nearly 90% of all diabetes cases. Even with the availability of medications, glucose monitoring, and insulin therapy, chronic complications include kidney failure, blindness, amputations, and even death. The insulin-producing beta-cells affected by diabetes are located within the islets of Langerhans. Islets are natural, small cell clusters scattered throughout the pancreas. They play an essential role in monitoring blood glucose levels and secreting hormones accordingly. Islets are composed of a variety of hormone-producing cell types including glucagon (alpha-cells), insulin (beta-cells), somatostatin (delta-cells), and pancreatic polypeptide (PP-cells). The insulin producing beta-cells make up a majority of the islet [56].

The challenges associated with drug discovery for those working in the field of diabetes have been immense. Insulin injection is a requirement for those suffering from type 1 diabetes as they lack insulin production. With 90% of diabetes patients suffering from type 2 diabetes, they make up the largest portion of the

diabetes drug market. According to one market research firm, the global market for diabetes pharmaceuticals was nearly $40 billion in 2010 and is projected to grow to $114 billion by 2016 [57]. Type 2 patients often require oral medications, sometimes combined with insulin, for glucose control. Current major types of medications include: sulfonylureas, meglitinides, biguanides, thiazolidinediones, DPP-inhibitors, and incretin mimetics (Table 2.1). Most of these oral medications act by increasing insulin output from islets, increasing insulin sensitivity on target organs, or decreasing glucose uptake into the blood stream. The side effects that accompany some of these drugs can include severely low blood glucose (hypoglycemia) and weight gain. Unfortunately, these therapies are still unsatisfactory at supporting glucose control, even in cases where multiple therapies may be employed at once [58-63].

Why has the pharmaceutical industry been so slow at bringing new diabetes drugs to the market? It has not been for a lack of trying, but rather the lack of suitable technologies. Current screening methods for diabetes drugs includes 2D islet cell culture using insulin-secreting beta-cell lines [39,64-66]. Monolayer culture is not natural for islet cells—or most cells for that matter. When islets are dispersed into individual cells and plated as a monolayer, the various cell types lose their inherent functions. This "senescence" is a direct result of the disruption of the inherent cell-to-cell contact present within intact islets, and has been known for decades to be vital in maintaining function of the islet as a whole [67-77]. Unless these cells are transformed or mutated for monolayer cell culture, loss in function is inevitable.

This provides much of the current reasons for the use of beta-cell lines instead of primary cells. In order for beta-cells to maintain insulin secretion in culture, cells are transformed, and stable cell lines are created. Beta-cell lines are mainly rodent-derived from insulinomas generated in transgenic mice or radiation-treated rat cells, and only a few cell lines exhibit glucose-sensitivity [39,66,78]. Genetic manipulations that arise when transforming the cells to a cancer-like phenotype can also raise new problems including the accidental destruction of native cell functions. The main goal in creating all of these cell lines is to produce insulin-secreting cells that retain similar characteristics to beta-cells. The use of genetically manipulated cells may produce new issues, especially with the native functional pathways being altered [39,66]. For instance, the transformed beta-cells may provide insightful knowledge into cellular mechanisms of cancer production, however they are less than ideal for the discovery of novel compounds for standard diabetes treatment.

As researchers continue to focus efforts on producing novel beta-cell cell lines, one might ask: what about the other cell types present within the islet? Within our bodies, islets exist as a cluster of various cell types. Extensive research supports that the combined presence of all cell types within an islets are involved in its *in vitro* and *in vivo* function [68,69,71-73,79]. Islets, once isolated from the pancreas, are used extensively in research and transplantation. Unfortunately, this is not necessarily the case of drug discovery in diabetes. It would make sense to use islets in their native 3D architecture, however 2D beta-cell monolayers are still in use today.

Native islets are small cell clusters that can be isolated from the rest of the pancreatic tissue. However, isolation leads to a variety of inherent problems. After isolation, the native blood supply is no longer intact, leaving diffusion as the primary mode of nutrients reaching the core cells. Within 24 hours of isolation, the limited diffusion of oxygen within islets leads to hypoxic or necrotic cores that result in diminished *in vitro* survival and function [80-82]. Adding to the issue, islets exist in a variety of sizes, ranging from 50 to over 400 μm in diameter. Glucose has been shown to diffuse with little effort into small islets (diameter < 125 μm), however can only penetrate the outer most cell layers of large islets (diameter > 150 μm) [82]. These studies go on to reveal several functional differences between small and large islets [56,80-84]. This variability in size (meaning large variations in cell numbers/islet), and variability in response to agonists greatly hampers any attempts to use intact islets for drug screening. For now, the use of native, intact islets is relegated for conducting secondary screens on promising hits and during lead optimization, but is not regarded as an appropriate primary screening tool.

CONCLUSION

In moving forward with developing new technologies for drug development, an analysis of the current status impresses upon one theme: there is a need to develop a physiologically relevant alternative for diabetes drug testing. When developing these technologies there are several points to consider for obtaining more clinically relevant results (Table 2.2). Novel cell-based assay technologies for diabetes drug discovery and HTS must be easily translatable to the commercial

sector while maintaining ease of use and minimizing additional capital expenditure. As we evolve into the next generation of drugs and therapeutics for diabetes, it is time to leave behind the disadvantages of the flat world of 2D monolayer cultures of the past and embrace the transition to organotypic 3D cell culture for the future.

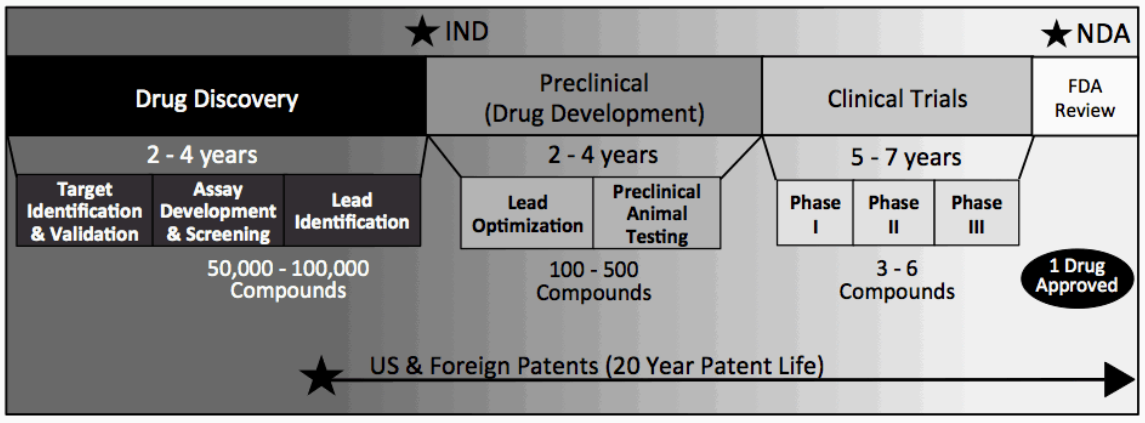


Figure 2.1: Drug Discovery and Development Timeline

The entire process from drug discovery through bringing a single drug to market can take 10 to 15 years. Patents are generally filed after a quality lead compound has been identified to protect the intellectual property and provide exclusivity for a 20-year period from the date of application. An investigational new drug (IND) application is filed with the FDA during preclinical trials to allow researchers to transfer the drug across state lines for further development. Once an IND is granted, the drug can move into human clinical trials. After the compound successfully progresses through clinical trials, a new drug application (NDA) is filed with the FDA to seek approval for manufacture, sale, and marketing.

Table 2.1: Anti-diabetic medications for the treatment of diabetes.

Medication*	Action	How they were discovered?	Currently Available Examples	Patent Expiration**	Side Effects	Available Drug Combinations
Sulfonylureas	Increase insulin secretion	Synthetic Chemistry. These are all sulfonylurea analogs.	*Second Generation*: Glipizide (*Glucotrol*) Glibenclamide (*DiaBeta, Glycron, Glynase, Micronase*) Glimepiride (*Amaryl*) Gliclazide (*Diamicron*)	All Generic	Hypoglycemia, Hyperinsulinemia, Weight Gain, Teratogenicity,	Metformin + Glibenclamide (Glucovance) Glimepiridine + Rosiglitazone (Avandaryl)
Meglitinides	Increase insulin secretion	Nateglinide discovered by screening amino acid libraries. Repaglinide developed via synthetic chemistry.	Nateglinide (*Starlix*) Repaglinide (*Prandin*)	Generic 2018	Hypoglycemia, Weight Gain	Repaglinide + Metformin (PraniMet) Can also be used in combination with thiazolidinediones, α-glucosidases, metformin, exenatide
Biguanides	Suppress glucose production by the liver	Biguanides originally discovered in 1922 from French lilac (folk medicine). Metformin developed via synthetic chemistry in 1922.	Metformin (*Fortamet, Glucophage, Glumetza, Riomet*)	Generic	Nausea, Bloating, Flatulence, Diarrhea, Abdominal Discomfort, Lactic Acidosis	Often prescribed in combination with meglitinides, some sulfonylureas, thiazolidinediones, DPP-inhibitors, or insulin.
Thiazolidinediones	Helps body respond to insulin (Insulin Sensitizer), can also lower glucose production by the liver	Accidental discovery of PPARs lead to the discovery of Thiazolidinediones as ligands.	Rosiglitazone (*Avandia*) Pioglitazone (*Actos*)	2012 2012	Edema, Weight Gain, Heart Failure, Heart Attack, Stroke	Metformin + Rosiglitazone (Avanade) Glimepiridine + Rosiglitazone (Avandaryl) Can be combined with insulin.
α-Glucosidase Inhibitors	Slows breakdown of starches and some sugars	Acarbose discovered from a natural product target-based screening in the 1970s. Miglitol created though synthetic chemistry.	Miglitol (*Glyset*) Acarbose (*Precose*)	Generic Generic	Nausea, Bloating, Flatulence, Diarrhea, Abdominal Discomfort	
DPP-inhibitors (dipeptidyl peptidase-4 inhibitors)	Increase in incretins including GLP-1 (glucagon-like peptide-1) and GIP (gastric inhibitory peptide) that allows increased insulin release	Sitagliptin discovered through the optimization of a class of β-amino-acid-derived DPP-4 inhibitors. Saxagliptin, Linagliptin, Vildagliptin created via synthetic chemistry.	Sitagliptin (*Januvia*) Saxagliptin (*Onglyza*) Linagliptin (*Tradjenta*) Vildagliptin (*Galvus*)	2017 2021 2017 2022	Nasopharyngitis, Headache, Sore Throat, Skin Irritation, Nausea	Sitagliptin + Metformin (Janumet) Saxagliptin + Metformin (Kombiglyze) Linagliptin + Metformin (Jentadueto) Vildagliptin + Metformin (Eucreas)

Table 2.1: (continued)

Medication*	Action	How they were discovered?	Currently Available Examples	Patent Expiration**	Side Effects	Available Drug Combinations
Incretin Mimetics (Injection)	GLP-1 and GIP analogs that lead to increased insulin release	Exenatide was discovered as natural protein from saliva of Gila monster. Liraglutide is a modified human GLP-1 analog.	Exenatide (*Byetta*) Liraglutide (*Victoza*)	2016 2017	Nausea, Vomiting, Headache, Diarrhea, Kidney Damage/Failure	Can be combined with thiazolidinediones, or insulin.
Amylin Mimetics (Injection)	Slows gastric emptying (appetite suppression) and suppress glucagon (Also works for type 1 diabetes)	Amylin (Islet Amyloid polypeptide) was found to be cosecreted with insulin.	Pramlintide (Symlin)	2019	Nausea, Headache, Decreased Appetite, Abdominal Pain, Tiredness, Dizziness	Used with insulin injection.

* Oral medications unless noted otherwise.

** Represented year is the earliest that a patent may expire and allow availability of generics. There is the possibility that additional method of use patents could be filed or further litigation by the manufacturer could increase the time of exclusivity.

Table 2.2: Factors to consider in 3D islet-like spheroid cell-based assay design for HTS and drug discovery.

3D Islet-Like Spheroid
✓ Primary Human Tissue
✓ Uniform 3D Size Range
✓ Normal and Uniform Drug Response
✓ Reliable EC50*
✓ Organotypic Composition/Structure
✓ Low Diffusion Barrier
✓ High Viability
✓ Ample Commercial Yield
✓ Compatibility with HTS Equipment
✓ Cost Effective Deployment
✓ Commercial Ease of Use

*ED50 = Half Maximal Effective Concentration

CHAPTER 3: Geometric Constraints During Islet Cell Reaggregation Using a Micromold.†

ABSTRACT

Using two-dimensional beta-cell monolayer cultures in high-throughput screening is insufficient for discovering new diabetes drugs. Lacking physiological relevance, beta-cells monolayers lack the complexities of native islets, and are not sufficient for accurate prediction of lead compounds during pharmaceutical drug discovery. Inherent size-dependent limitations within native islets can alter data and lead to erroneous conclusions. The superior viability and functionality of small islets (diameter < 125 μm) in comparison to large islets (diameter > 150 μm) demands a look at engineering an appropriate solution. To engineer islets while limiting size (below 125 μm) we designed a glass micromold containing conical-shaped micro-wells. Within these wells, dispersed islet cells spontaneously reaggregate into islet-like spheroids within the geometric constraints of the well. The glass micromolds can be reproducibly fabricated in a variety of shapes and sizes. The high efficiency production of engineered islet, or Kanslets, remained within our size limitations. Using the micromold lead to high commercial yields, producing on average 20 Kanslets per mm^2. In high-throughput settings, native islets posed problems for standard equipment, however Kanslets were dispensed with high well-to-well efficiency. Having a technology to produce islet-like spheroids can provide a superior model for diabetes drug discovery. The glass

† In preparation as **Ramachandran, K.**, Stehno-Bittel, L. "Geometric constraints during islet cell reaggregation using a micromold," for Biofabrication, 2012.

micromold provides a scalable technology for user-friendly, high-efficiency production of 3D cell spheroids in the commercial marketplace.

INTRODUCTION

Diabetes affects 8.3% of the populations within the United States and over 346 million individuals worldwide [52,85]. The economic burden within the United States alone exceeds $200 billion and demands the need to do research and develop new and successful therapies. As the pharmaceutical industry continues to focus efforts on diabetes drug discovery, the currently available technologies are insufficient and tools for discovering the next generation of drugs is limited. A potential contributor to this issue is the extensive use of 2D beta-cell monolayer culture as the primary tool for modern drug discovery.

The complex three-dimensional (3D) cellular environment is essential in supporting the structure and function of cells within our bodies. This environment provides support to intracellular communication, mechanical stimulation, biochemical signaling, and plays a key role in the overall function of the tissue as a whole. As we look toward innovating 3D cell-based solutions, we must remain true to the natural behavior of cells and strive to engineer those that best mimic the *in vivo* environment. The development of 3D cell culture allows for physiological significance when providing tools for drug discovery and regenerative medicine. As researchers transition to 3D cell culture, we begin to bring relevance between traditional cell culture techniques and living systems.

Whether in a cell-based assay for primary screening in a high-throughput screen (HTS) or bench top *in vitro* experiments, using beta-cell monolayers limits the possibility to accurately and predictably discover new medicinal entities (NMEs) to treat diabetes. Extensive research in the field demonstrates that non-transformed, dispersed islet cells lose their ability to respond to glucose with insulin secretion when grown in monolayers [39,67,72,73,78]. Genetic modifications create transformed beta-cell lines that unfortunately raise more concerns than they solve [39]. Islets are much more complex micro-organs composed of a variety of cell types including alpha-, beta-, and delta-cells (glucagon-, insulin-, somatostatin-secreting cells; respectively). These small, multi-cellular clusters, scattered throughout the pancreas, play a vital role in blood glucose level maintenance. The paracrine signaling between the various islet cell types further supports the structure-function relationships that is essential to natural islet function [68,72,86]. Thus, when finding drugs that treat diabetes, using beta-cell monolayers for drug screening lacks relevance in a drug's action on a more complex system.

Unfortunately, isolated islets provide their own challenges. Isolating islets from their native location in the pancreas has a profound effect on oxygen and nutrient delivery, ultimately leading to core cell death and decreased function. With the absence of the native blood supply, diffusion becomes the primary means of transport of small molecules into an islet [82,87]. Islets can exhibit core cell death within 24 hours of isolation, leading to reduced function and eventual islet loss [80]. The size of islets has been demonstrated as a contributing factor toward survival and function. More specifically, small islets (diameter < 125 μm) show superior

functional performance and increased insulin production as compared to large islets (diameter > 150 μm) [56,80-84]. Mathematical modeling supports this finding by demonstrating the limited diffusion of oxygen into the core of islets having a diameter larger than ~100 um [82]. When perifused with fluorescent glucose, size-dependent penetration of the islet core was witnessed [82].

In order to provide a more suitable alternative, the engineering of optimal islet-like clusters could overcome the diffusion barriers affected by islet size limitations. Although dispersing islets into single cells would greatly decrease any diffusion barriers, as mentioned previously, dispersed islet cell monolayers show decreased function. The intracellular interactions are vital to islet phenotypic maintenance [67,69,72-76]. For several decades, researchers have witnessed the spontaneous reaggregation of dispersed islet cells and have attempted to utilize this natural phenomenon in reconstructing islet-like tissues [88-93]. In the early forms of "pseudo-islet" formation, islet cells were incubated in culture and allowed to reaggregate into structures of varying shapes and sizes. Often times, this technique resulted in islets larger than 200 μm. The problem with this methodology is the regeneration of large cell clusters—reintroducing the diffusion barrier, inferior viability, and reduced function. Understanding the size-dependent limitations within islets, engineering islets of a small size (less than 125 μm) is necessary to prevent restoration of these inherent issues.

Researchers are developing new techniques and technologies for engineering islets of a defined size, however many fail to catch on due to complications or problems within their respective approach. Many gravity-enforced techniques,

including the hanging drop method, have been widely used to produce and study 3D cell spheroids, primarily in stem cell and cancer biology [38,94-98]. In the hanging drop method, cells are suspended within culture medium in a drop-wise fashion and undergo gravity-enforced aggregation. Although this method allowed aggregate formation, it came with the excessive manual labor of individually placing drops on a petri dish lid with an inability to scale up for mass production. Today, researchers are developing tools to automate and increase the production of hanging drops [99-101].

These techniques surely improve upon the original formulated method, are automated, and claim their suitability in a HTS setting, but they still lack scalability and present new challenges. Current technologies for HTS aim for ultra-high throughput (1536-wells or more per plate) and are using smaller solutions such as microarrays [17,25,42,102,103]. The development of 384-well hanging drop plates allows HTS automation capabilities, but when moving forward into ultra-high throughput, scalability becomes an issue. Culture medium changes within these plates require careful removal and dispensing of media in very small volumes within each individual hanging drop. Furthermore, a humidifying water-reservoir is required to prevent evaporation of hanging droplets and valuable tissue loss. These extra steps increase the complexity for screens, increase costs, and simply do not present valid solution for the future.

Developing a tool for the production of organotypic islet cell spheroids that are geometrically constrained, compatible with HTS automated equipment, and can be produced at a high output can have considerable impact on diabetes drug

discovery and 3D cell culture. Next generation 3D cell culture technologies should be readily accessible and easy to incorporate into the already expensive HTS infrastructure. Additionally, they should be easy to tailor for future technologies as industry standards change. Developing a high quality solution should reduce the cost of employing 3D cell culture in various processes and increase industry adoption.

In this study, we have developed a technology to produce geometrically optimized islet-like spheroids with increased scalability through high commercial yield and compatibility with drug screening high-throughput equipment. The primary focus in developing this technology was limiting the diameter of islets during reaggregation to no bigger than a small islet (diameter less than 125 μm). These engineered islets were produced *in vitro* through the reaggregation of dispersed, isolated islets on a glass micromold containing micro-recesses of a defined geometry. This provided a tool that was sterilizable, reusable, transparent for microcopy and high-content analysis, and allowed reproducible engineering of optimized islets, or Kanslets. In doing so, we have provided a tool and method for effectively engineering islet-like clusters with geometric constraints (based on previous size-dependent behavior) and provided a surface for high-output production and cell culture of 3D tissue spheroids. Kanslets can easily be removed from the micromold and used for diabetes drug testing and other *in vitro* experimentation. In a regenerative medicine application, Kanslets can be investigated as an alternative to using isolated, native islets for islet transplantation in treating diabetes.

MATERIALS AND METHODS

Rat Islet Isolation

Rat pancreatic islets were isolated and dispersed using our previously published procedures [80,82,104,105]. Briefly, rats were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg; Ketaject, Phoenix Pharmaceuticals, St. Joseph, MO) and xylazine (10 mg/kg; AnaSed, Lloyd Laboratories, Shenandoah, IA). After the peritoneal cavity was exposed, the main pancreatic duct to the duodenum was clamped, cannulated *in situ* via the common bile duct, and distended with cold collagenase type 1 (CLS1; Worthington, Lakewood, NJ). After excision, the pancreas was incubated for 20-30 minutes with gentile tumbling in a 37°C incubator. The contents of the tube were washed, passed through a 100 μm mesh screen, and sedimented in a refrigerated centrifuge. The pellet was mixed with Histopaque (density = 1.1085) and centrifuged. The islets collected from the gradient were sedimented and washed over a sterile 40 μm mesh cell strainer. Islets were placed into DMEM:F12 or CMRL containing 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic and allowed to recover overnight in an incubator at 37°C and 5% CO_2.

Human Islet Procurement

Human islets were procured via overnight courier through the National Institutes of Health Integrated Islet Distribution Program. Generally, after receiving a shipment, islets were incubated overnight in CMRL media at 37°C and 5% CO_2.

Micromold Design and Fabrication

Disc-shaped, soda-lime glass substrate, were ordered from Precision Glass and Optics (Santa Ana, CA). The glass substrates were cleaned for 20 minutes each in an acid piranha solution (70:30 H_2SO_4/H_2O_2) followed by a base piranha solution (70:30 NH_4OH/H_2O_2) to remove any contaminants. Glass substrates were subsequently dried at 200°C for 30-60 minutes to ensure that the surface was free of moisture.

Glass micromolds were fabricated through a multistep process that used thin-film deposition, photolithography, and wet etching techniques (Figure 3.1). Clean glass substrates were placed in a Lesker Thin Film Deposition System. A 300 nm layer of chromium was sputtered onto one side of the disc. Next, a positive photoresist (AZ1518) was spin-coated onto the chromium-coated surface to yield a 1.8 μm layer. Photoresist-coated discs were prebaked at 100°C for 2 minutes. A transparency mask (photomask) template was created containing spots of a defined shape, size, and layout using computer-assisted design (CAD) in AutoCAD software (Autodesk) and high-resolution transparency masks were printed. The photoresist-coated substrate was exposed to UV light through the photomask for 4 seconds. Exposed glass was postbaked at 100°C for 10 minutes and then immersed in AZ 300 MIF Developer to render the image. The chromium layer was etched with the pattern by immersion in CR7S Chromium Etchant. Glass was washed with water and dried with nitrogen gas.

To etch the pattern into the surface of the glass, the disc was wet etched by immersion in a buffered oxide etch (BOE) solution containing 14:20:66

$HNO_3/HF/H_2O$. Immersion time varied from 10-20 minutes depending on the intended etch rate and the freshness of the BOE solution. Due to isotropic etching the recess depth and diameter were optimized through a combination of template design, immersion time, and etch rate. After immersion in the BOE solution, glass was washed with a calcium carbonate slurry to neutralize HF reactivity. A profilometer (Tencor Alphastep 200) was used periodically to measure the etched surface and adjustments in immersion time were made accordingly. The photoresist and chromium layers were then removed using acetone and chromium etchant respectively. Glass was washed with water and dried with nitrogen gas.

To contain the cell culture on top of the glass micromold, a large polydimethylsiloxane (PDMS; Sylgard 184; base and cross-linker; Dow Corning, Midland, MI) well was molded to surround the glass disc. PDMS is a silicone-based polymer that is translucent, sterilizable, and often used in experiments in direct contact with cells [106]. The base and curing agent were mixed at 10:1 ratio (base to curing agent) and deposited into a copper mold. The PDMS was cured in an oven at 60-100°C for 1 hour. All micromold components were cleaned and sterilized prior to any cell culture or *in vitro* use.

Islet-Like Spheroid Production

Kanslets were produced by dispersing isolated, native islets into single cells, loading onto the micromold, and culturing for several days until reaggregation occurred. Isolated islets were dispersed into single cell suspensions by placement in a 1.5 ml eppendorf tube. Islets were washed twice with calcium- and magnesium-

free HBSS (cmf-HBSS) before addition of digestion medium containing cmf-HBSS supplemented with 4.8 mM HEPES and papain (5 units/ml; Worthington, Lakewood, NJ). Tubes were incubated on a rotator at 37°C for 20 minutes. Islets were dispersed by trituration using a pipette until the cell suspension primarily contained single cells. The papain reaction was stopped by adding complete media containing calcium, magnesium, and FBS. The cells were then washed to remove residual papain and transferred to a Kanslet aggregate culture medium. The Kanslet aggregate medium consisted of D-MEM:F12 or CMRL1066 supplemented with 10% fetal bovine serum (FBS), and 1% Anti/Anti. Occasionally, samples were taken to determine cell counts and yield.

Micromolds were placed into sterile petri dishes with lids for incubation and to prevent contamination. The single cell suspension was then carefully plated onto sterile glass micromolds. A total volume of 3-4 ml of aggregate medium was used and maintained to minimize diffusion issues due to culture medium height. Micromolds were incubated for 5-7 days at 37°C and 5% CO_2. Aggregate culture medium was changed every 24-48 hours until reaggregated islets were formed. Kanslets were removed by washing the micromold several times with culture medium until islets dislodged and then aspirated with a pipette. Kanslets were maintained in DMEM-based or CMRL-based culture medium.

High-Throughput Dispensing

A known concentration of islets and Kanslets were suspended in culture medium and dispensed into 384-well plates using a Matrix WellMate Microplate

Dispenser (Thermo Scientific) at the High-Throughput Drug Screening Laboratory at the University of Kansas, Lawrence. After dispensing, islets were counted manually using a microscope and a distribution map of islet dispensing was created using Microsoft Excel software.

Volume Measurements and Statistics

Images were obtained using a Nikon inverted light microscope with attached camera or an Olympus confocal microscope housed at the Diabetes Research Laboratory at the University of Kansas Medical Center. Images were acquired at 10X-100X an analyzed using Olympus FluoView, Adobe Photoshop, or Lumenera Infinity Analyze.

Islet volumes were calculated by measuring the islet diameter and then converting it into islet equivalents (IEQ) or cell number [105]. A hemocytometer was used to estimate cell number when islets were dispersed in a single cell suspension. Yield was calculated by comparing IEQ or cell number before islet dispersion and after Kanslet production. Islet diameter was measured using an ocular micrometer, FluoView Software, or Infinity Analyze. We measured the diameters of Kanslet samples produced over various preparations to determine the production uniformity between preparations. Several samples were taken from each preparation, diameters were measured and a size distribution calculated. When counting spheroids, those with a diameter less than 25 μm were disregarded.

Measurements were performed in duplicate or more, with results calculated as averages ± standard deviation. Statistical significance was evaluated using a Student's *t*-test with significance defined as $p < 0.05$.

RESULTS

Overall, the micromolds were simple to use for tissue spheroid production (Figure 3.2). After islet cells had been dispersed into single cells, the cell suspension was loaded directly on top of a micromold, and after several days Kanslets were produced. Within 24 hours of seeding, cells had settled into wells and very loose aggregates had started to form. Over the next several days, loose aggregates coalesced into a tighter conformation and developed an islet-like morphology (Figure 3.3). Removing Kanslets from the micromold is as simple as washing the plate with culture media several times and aspirating the spheroid containing solution with a pipette. Retrieval efficiencies were rarely a problem and less than 0.1% were left behind.

We were able to reproducibly fabricate micromold discs (33 mm diameter) with an average 20 micro-wells per mm^2 (Figure 3.4). The discs were originally designed to fit into standard 6-well plates (Figure 3.4A). Frequently, cells that were loaded on top of the wells would end up beneath the glass (Figure 3.4B). This was compounded by the design requirement to prevent the excessive labor of changing media in individual wells by simply covering the entire micromold well surface in culture media. To keep with this requirement, a PDMS wall was molded to surround the disc, creating a self-contained micromold (Figure 3.4C). In the self-contained

version, changing culture media took replacing 50% of the media in a single step. Culture medium was changed at a slow rate to prevent un-aggregated cells or spheroids from shifting.

The micromold itself contains conical-shaped micro-wells were 200-240 μm in diameter and 70 μm deep. The design process underwent several iterations before resulting in success. A modified multi-layer approach using a chromium-layer and photomask, allowed increased well depth (70 μm) compared to using a photomask alone (< 5 μm) [107,108]. In addition to a deep etching strategy, well placement became crucial (Figure 3.5). In an early design, wells contained spaces (~125 μm) between each other (Figure 3.5A). These micromolds contained 8650 wells on a 33 mm diameter disc (10 wells per mm^2). We were able to successfully produce Kanslets using this layout, however many times we found that cells settled in the space between wells. Although subsequent movements, media changes, and fluid flow shifted some of these cells into wells, additional single cells remained separately on the surface.

In the current design, wells are placed in close proximity, increasing the probability cells would fall into wells (Figure 3.5B). We were able to decrease the space between wells to less than 20 μm, ensuring that wells did not merge together. Using these micromolds, all loaded cells settled into the wells leaving little or no cells in the remaining space. This micromold version contained over 15,000 wells on a 33 mm diameter disc. When well sizes were on the smaller end (~195 μm diameter), micromolds contained up to 23,000 wells in the same area.

To determine the production efficiencies we calculated a commercial yield based on the number of Kanslets that were produced on a single micromold during a preparation. We averaged a commercial yield of 16483 ± 6384 Kanslets per micromold. This is significant because we are able to produce more Kanslets per area compared to other methods (Figure 3.6). Compared to a 384-well hanging drop plate (producing only a single spheroid per well; less than 1 spheroid/mm^2), we exceed the production capacity by 43 times (19 Kanslets/mm^2). The micromold in our examples are only 33-mm in diameter, although glass micromolds can be fabricated in a variety of shapes and sizes. If micromolds were built to the same area specification as standard 384-well hanging drop plates or potentially developed 1536-well hanging drop plates, we could increase production to 200,000 spheroids (520 times more, 130 times more; respectively).

Since our goal was to produce spheroids that were below 125 μm in diameter, we defined uniformity as the production of spheroids in a range between 25 and 125 μm. On occasion, we found that some wells contained multiple aggregates instead of a single Kanslet. Since these aggregates were still within the size limit, they were considered as Kanslets. In general, we were able to produce Kanslets with an average diameter of 49 ± 25 μm with 99.2% having a diameter less than 125 μm (n = 3501 Kanslets). The size uniformity between various Kanslet preparations remained consistent with diameters averaging 49 ± 2 μm (standard error) (Figure 3.7). The production uniformity remains evident in comparison to native islets, which have a size range of 50 μm to over 300 μm (Figure 3.8).

Using Kanslets can provide a lot of benefits for commercial use in a high-throughput setting in comparison with attempts to use native islets. In our experimental commercial run, we attempted to dispense native islets (2 per well) and Kanslets (20 per well) into 384-well plates using automated dispensing instruments common in commercial drug screening. These dispensing numbers were chosen in order to dispense similar tissue volume per well (Table 3.1). Calculating islet volume allows for the normalization of 3D islet structures. Volume normalization was calculated by measuring islet diameters, then converting them to either islet equivalent (IE) or cell number [105].

When working with intact native islets, the instrument dispensed 2.5 ± 1.8 islets per well (range: 0 to 9 islets per well). Unfortunately, over 10% of the wells were empty, dispensing media without an islet (Figure 3.9). To generate plates containing islets in all wells, we had to dispense a much higher concentration of islets. This produced an increased volume variation with 1 to 14 islets per well and an average of 4.9 ± 2.5 islets per well. When Kanslets were dispensed into plates, we achieved a 100% dispensing efficiency with an average 19.7 ± 5.8 Kanslets per well. This tighter range translated to an average 2277 ± 569 cells per well or 0.7 ± 0.4 IE per well.

DISCUSSION

The use of traditional 2D cell culture is rapidly transitioning to the world of 3D spheroids providing for greater relevance. Several new technologies are being developed to produce 3D cell clusters. Unfortunately, cost, ease of use, and

scalability are among the problems that hinder commercial acceptance of such products. In developing our micromolds, we have provided a technology that can easily be translated for commercial applications.

The use of beta-cell monolayers as an *in vitro* representation for natural islets is insufficient [39]. Introducing isolated, native islets as a solution may seem viable. However, it too comes with inherent issues [56,80,82-84]. Given these known issues, engineering optimal islet-like spheroids can produce an applicable solution for use as an alternative to beta-cell monolayers. When designing our technology, in addition to the geometric considerations, were several key factors enabling commercial use. These included (1) simple production of 3D spheroids, (2) high commercial yields, (3) use with current standard equipment for high-throughput screening, and (4) scalability for today and tomorrow.

Through a combination of micromold design and a multi-step fabrication process, we produced micromolds containing micro-wells of a defined geometry for optimally engineering islets. Engineering islets was made to be a simple process including simple cell seeding onto micromolds, subsequent cell culture with culture media changes, and easy removal of produced spheroids. Additionally, by using glass, micromolds were cheap, sterilizable, reusable, transparent, and compatible with *in vitro* cell culture.

The micromold was specifically designed for simpler use compared to currently available products. All of the wells within the micromold were placed on a single surface allowing simple loading of a cell suspension. This allowed cells to settle into wells and made culture medium changes simple. Some technologies are

faced with the inability to easily remove or retrieve spheroids after formation. In our case, we were able to simply wash the micromold, collecting over 10,000 spheroids from a single preparation.

Kanslets demonstrated a higher dispensing efficiency when utilizing automated dispensing systems to load high-throughput plates. Because Kanslets can be easily removed from the molds, they can be loaded using industry standard technology into commercially available plates. In a 384-well plate, we are able to maintain dispensing efficiency using Kanslets. Dispensing native islets remains inaccurate with well-to-well volume differences being large. This variability in volume remains large even if a single islet per well was dispensed. Dispensing Kanslets was more efficient in decreasing the well-to-well accuracy. Although we loaded 20 Kanslets per well, it should be possible to gain similar results at a smaller number per well. Simply put, since native islets have a broad size range, large and small islets will have different properties when flowing through liquid, and often times cannot be uniformly held in suspension for even dispensing. This islet size difference is most likely a key concern leading to variability in the well-to-well values.

From our experiences with native islets, we had to incorporate the use of a COPAS Large Particle BioSorter to purposefully load a specific number of islets per well. In fact, only one COPAS machine in Easter Kansas could be used with large native islets, as we frequently clogged the tips of most machines. The introduction of additional equipment and steps can become cumbersome, decreasing efficiencies in the entire process, resulting in increasing costs. Keep in mind that the nozzle tips

and dispensing volume used in many of these large systems are designed to accommodate a variety of well plates. When moving to smaller well sizes such as 1536-well plates and those being designed for ultrahigh-throughput screening, nozzles get smaller, liquid volumes are smaller, and dispensing larger object becomes more difficult.

Ultimately, we satisfied our primary goal by successfully producing islet-like spheroids while constraining diameter within a size range comparable to small islets (25 to 125 μm). Within several days of seeding, islet cells had reaggregated into tightly formed spheroids taking on an islet-like morphology. The demonstrated natural ability of islet cells to self-assemble into an islet structure enforces the need to transition away from 2D beta-cell monolayers. Though we successfully have produced an islet-like structure, our next step will be to characterize them in comparison to native islets in terms of functionality. As a whole, Kanslets contain the multiple cells types that make up native islets from which hey are produced. This means that when the cells are reaggregated, the new organotypic structure should mimic islet structure and function. In providing a comparison to native islets, we will need to examine if we have overcome the inherent issues within native islets. This characterization should examine cell composition, viability, glucose diffusion, and glucose-dependent insulin section.

This study was a basic proof-of-concept demonstration of the micromold technology in producing islet-like spheroids. Beginning with simple spheroid production, we present a tissue engineered solution to increasing the physiological relevance of cell culture. The 3D structure aims to replicate the structure found in

living systems, bridging the gap between *in vitro* experimentation, animal studies, and eventual human application. The significance of this technology can impact tissue engineering approaches for basic science, drug discovery, regenerative medicine, and more.

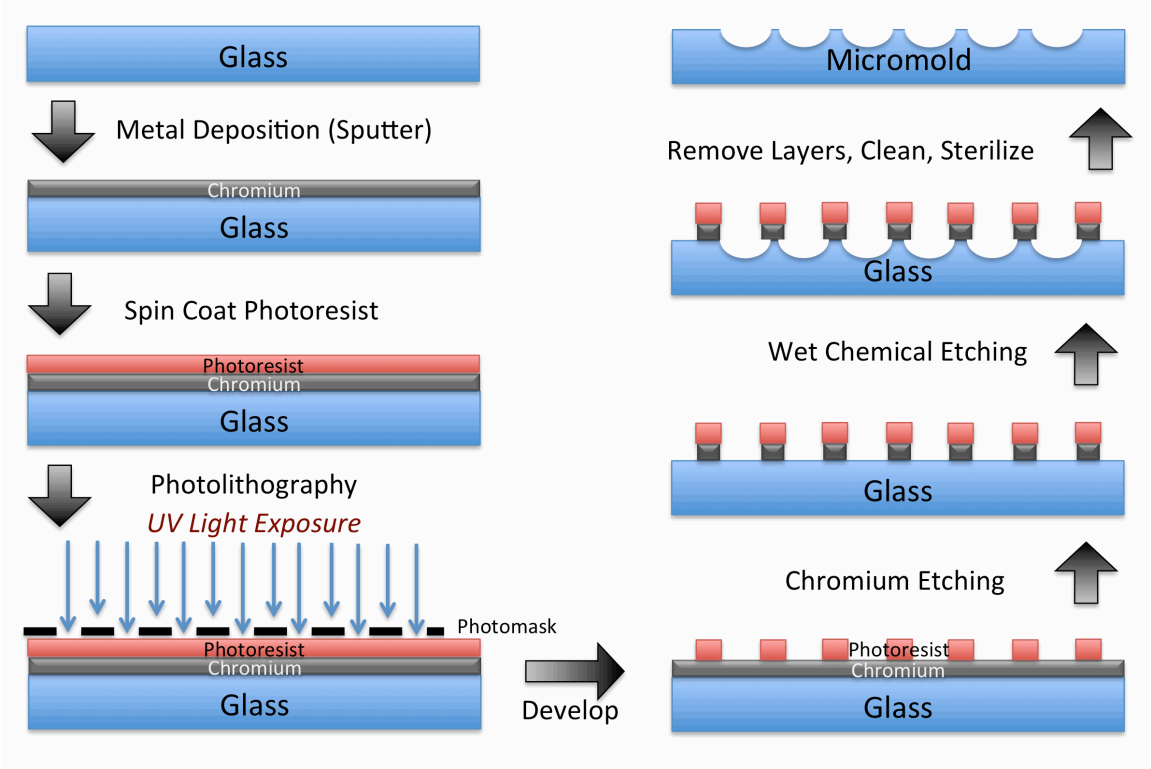


Figure 3.1: Fabrication of the Glass Micromold Surface

The micromold fabrication process for deep well etching consists of a multilayer approach consisting of chromium and photoresist. This multistep process requires a combination of metal deposition, photolithography and wet chemical etching to produce conical-shaped micro-wells on the glass surface.

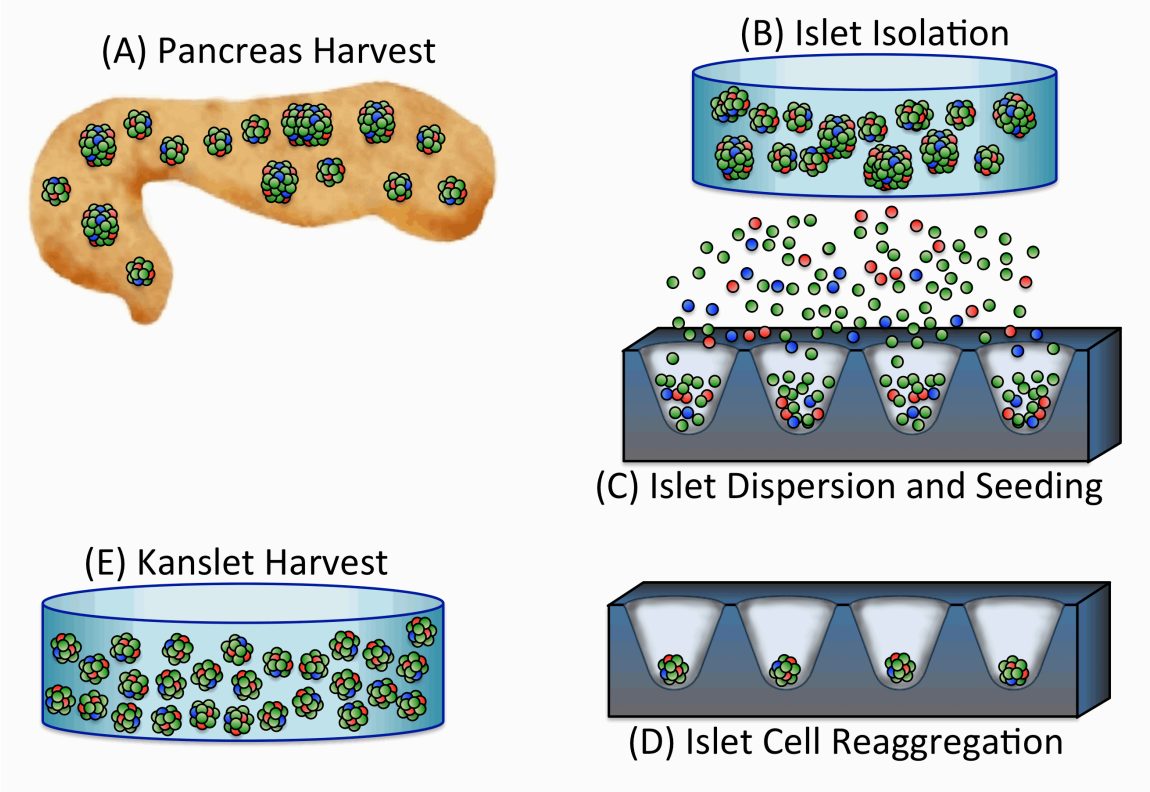


Figure 3.2: Simplified Islet Tissue Engineering Process

To produce Kanslets, a pancreas is harvested from the donor (A) and islets are enzymatically isolated from the exocrine tissue (B). Islets, composed of various sizes, are dispersed into single cells and seeded onto the micromold (C). Within several days islet cells reaggregate into size-constrained spheroids within micromold (D). Uniformly produced Kanslets are removed from the micromold for subsequent use (E).

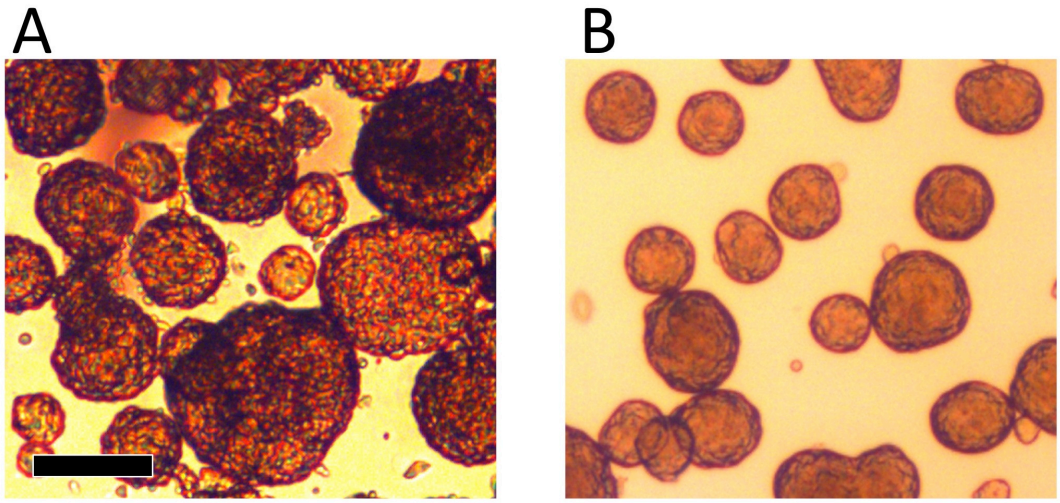


Figure 3.3: A Comparison Between Native Rat Islets and Uniform Kanslets

(A) Native rat islets representing a broad size range. (B) Uniformly produced rat Kanslets. Scale bar = 125 um.

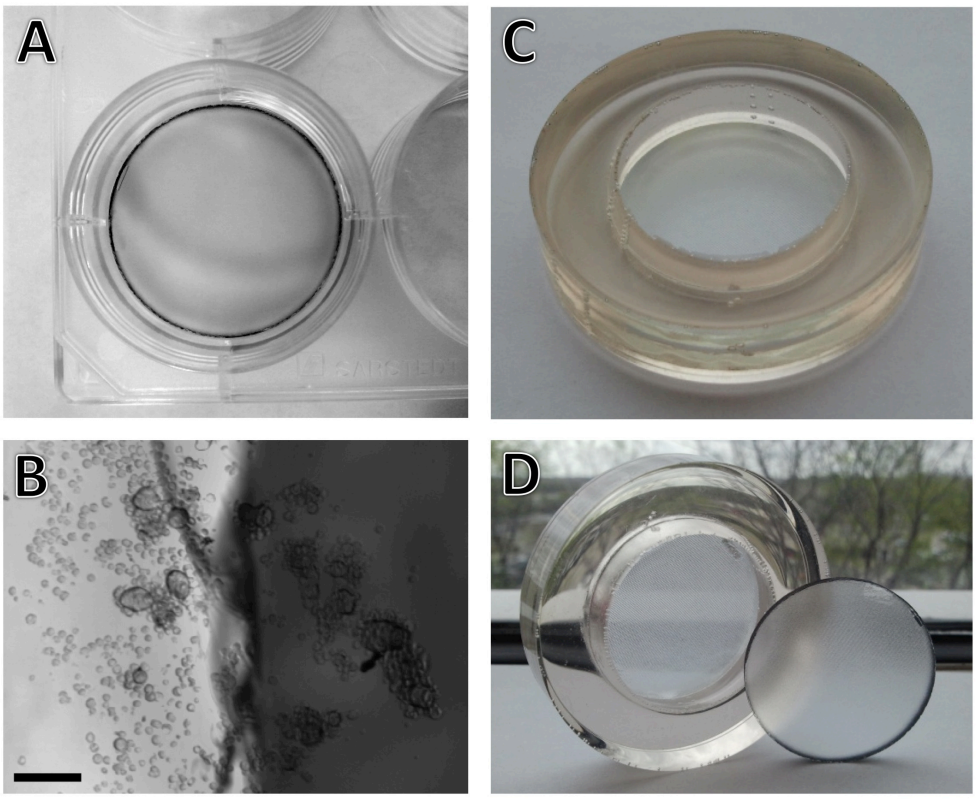


Figure 3.4: Glass Micromold and Self-contained Micromold

The glass micromolds in these images are all 33 mm in diameter. (A) A micromold in the bottom of a standard 6-well plate. (B) Without containment, aggregates and cells fall off the top surface of the micromold. (Scale bar = 200 μm). (C) A self-contained micromold consists of a glass micromold surrounded by a PDMS wall. (D) Another view of the glass disc alone next to a self-contained micromold.

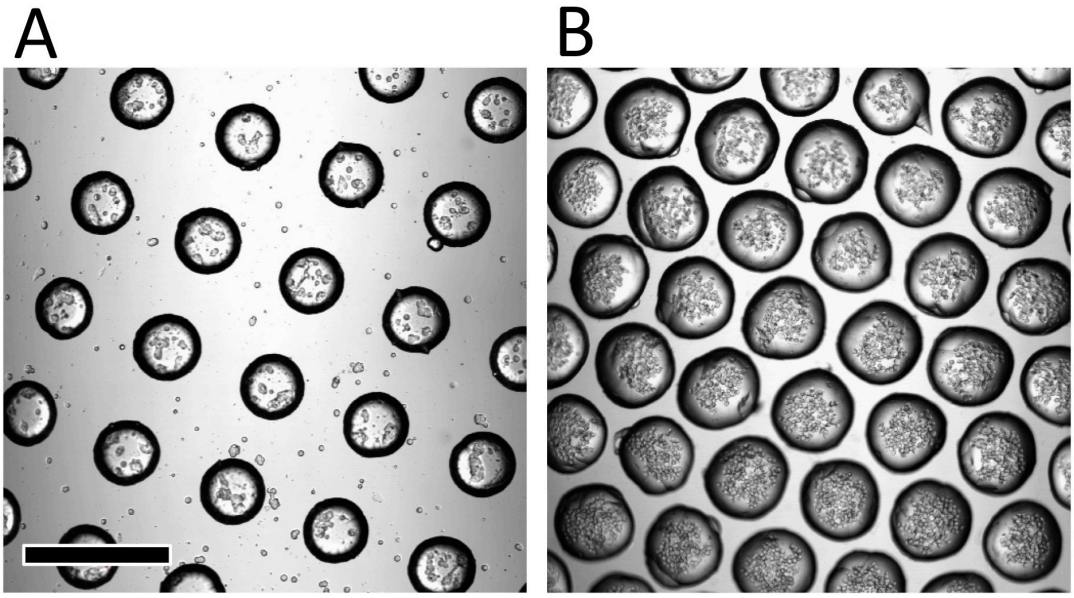


Figure 3.5: Various Micro-well Layouts

(A) Cells have settled between wells in this initial design consisting of micro-wells with large spaces between them. (B) With micro-wells in closer proximity, no cells can be seen settling between the wells. Scale bar = 200 μm.

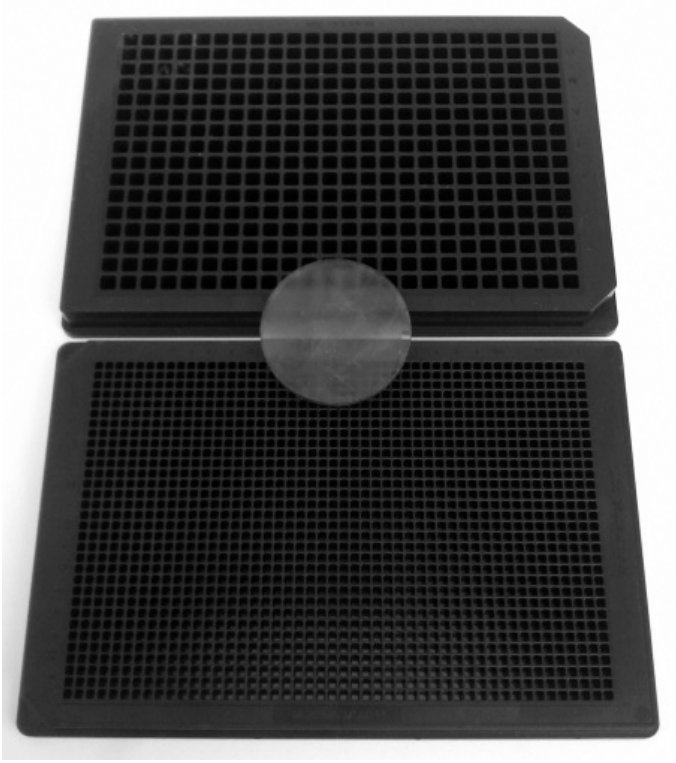
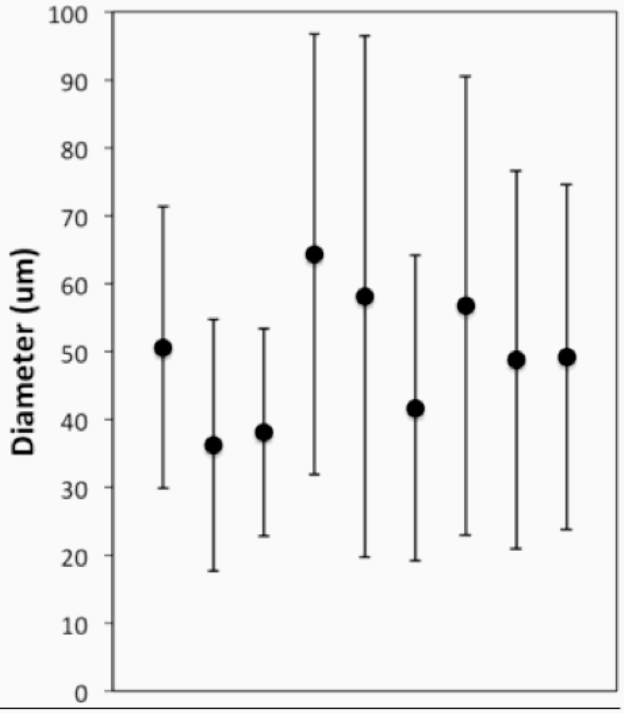


Figure 3.6: Micromold and Standard Well Plates

A glass micromold (33 mm diameter) containing over 20,000 wells is pictured with industry standard 384-well and 1,536-well plates.



Run #	Average Diameter (µm)	SD (µm)	% < 125 µm
1	49	25	98%
2	49	28	99%
3	57	34	98%
4	42	22	100%
5	58	38	95%
6	64	32	98%
7	38	15	100%
8	36	19	100%
9	51	21	100%

Figure 3.7: Uniformity Between Various Kanslet Preparations

Production uniformity was compared between various Kanslet preparations. This graph presents the average diameter with standard deviations. The table contains the individual values correspond to the graph from left to right. Between these individual preparations, Kanslet diameter averaged 49 ± 2 µm (standard error). (n = 1961 Kanslets in 9 replicates; error bars = standard deviation)

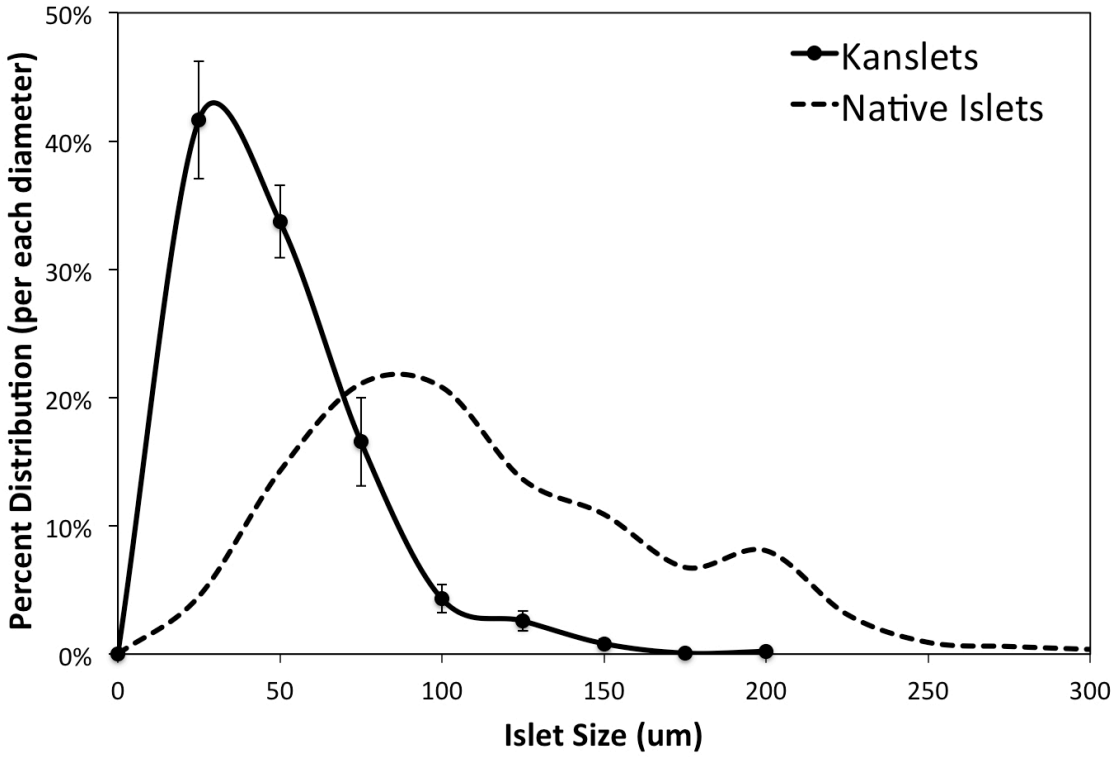


Figure 3.8: Overall Size Uniformity of Kanslets

The various Kanslet preparations were averaged to demonstrate the overall uniform production of Kanslets within a limited size range. Over various preparations, Kanslet production maintained uniformity below the 125-μm thresholds. (n = 1961 Kanslets in 9 replicates). Native islets exist in a broad size range (n = 5691 native islets). (Error bars: standard error)

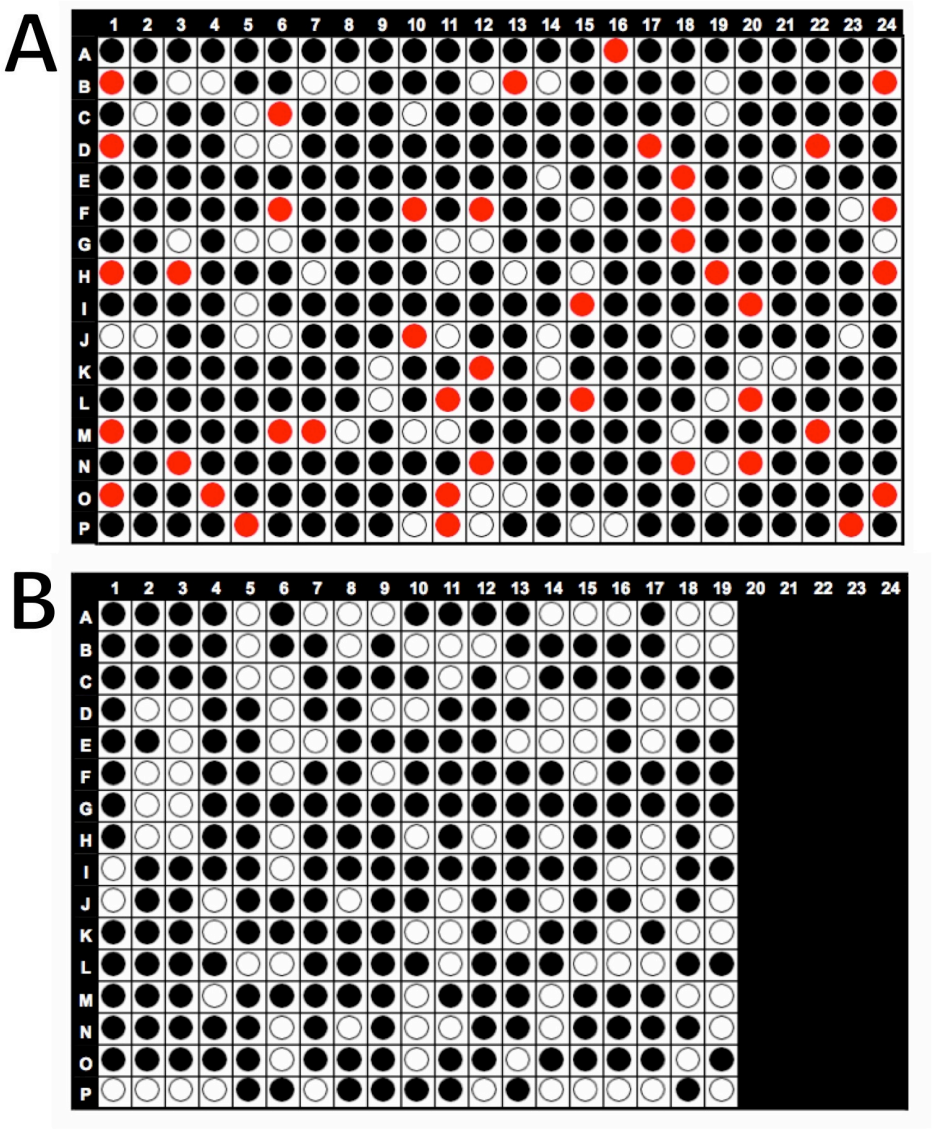


Figure 3.9: High-Throughput Dispensing into 384-Well Plates

Native islets (A) and Kanslets (B) were dispensed into standard 384-well plates using industry standard equipment. The number of islets per well was counted to prepare these representative figures. Red spots represent empty wells. Black spots represent the number of wells within 1 standard deviation of the mean number of islets for that plate. Due to the limited number of total Kanslets available during this experiment, we chose not to dispense into all columns.

Table 3.1: Theoretical calculations for high-throughput dispensing of islets and Kanslets.

Tissue Type	Typical Size Range (µm)	Number/Well	Theoretical Range (Calculated)	
			Islet Equivalent (IE)	Cell Number
Native Islets	25 - 300	2	0.01 – 16	144 – 7651
		20	0.07 – 160	1440 – 76505
Kanslets	25 - 100	2	0.01 – 0.59	144 – 745
		20	0.09 – 5.93	1440 – 7450

CHAPTER 4: Engineering Islets for Improved Performance by Optimized Reaggregation in a Micromold‡

ABSTRACT

Isolated islets can provide a source of tissue for research, transplantation and drug discovery in order to develop therapies for diabetes. Empirical modeling of islet diffusion barriers demonstrated that only the outermost layers of cells were exposed to glucose and sufficient oxygen levels, resulting in core cell death. Islets under a diameter of 100 μm exhibited a lower diffusion barrier, superior survival rates, and improved functional properties. Utilizing these observations we engineered optimal islets by dispersing them into single cells and reaggregating them over several days in a micromold. These custom-designed micromolds contained conical-shaped recesses that enhanced reaggregation of cells into a defined geometry. The engineered islets, or Kanslets, were all under 100 mm in diameter, and had the same general cellular composition as native islets. Kanslets continued to produce new insulin molecules and had microvilli on the islet surface, much like native islets. The engineered islets had a statistically higher viability (percent of live cells), and increased glucose diffusion compared to native islets. In addition, they remained responsive to varying glucose levels by secreting insulin. When transplanted into diabetic rats, engineered islets performed identical to native islets in reducing blood glucose to normal levels. Optimally engineering islets

‡Submitted with revisions as **Ramachandran, K.**, Williams, S.J., Huang, H-.H., Novikova, L., Stehno-Bittel, L. "Engineering islets for improved performance by optimized reaggregation in a micro-mold," for Tissue Engineering: Part A, 2012.

may be a suitable alternative to utilizing native, isolated islet tissue for a variety of applications. Reaggregating tissue in an optimized manner using our engineered micromold approach has immense impact for three-dimensional tissue production and its subsequent use in research, drug discovery, and the clinic.

INTRODUCTION

There are over 18.8 million individuals in the United States diagnosed with diabetes due to disregulation of blood glucose levels [52]. Islets of Langerhans are cell clusters within the pancreas composed of a variety of cell types including alpha-, beta-, and delta-cells, and are responsible for the maintenance of blood glucose level. Lymphocyte destruction of beta-cells (insulin-producing cells) or failure to utilize insulin are the hallmark events that result in type 1 and type 2 diabetes, respectively. Monitoring blood glucose levels and administration of insulin injections remain the primary therapies currently available to patients, requiring stringent patient compliance. Individuals suffer from chronic complications including kidney failure, blindness, amputations, and even death. There is an urgent need to provide better solutions as we move forward in treating this debilitating disease.

Isolating islets from the pancreas of cadaveric donors provides tissue that can be used for both transplantation and drug discovery. Significant strides have been made to offer pancreatic islet transplantation as a means to achieve insulin independence [109-113]. With the advent of the Edmonton protocol, recipients were able to maintain normoglycemia for a full year after islet transplantation

[114]. Unfortunately, regardless of these results, long-term success is limited and patients are subject to receiving multiple transplants to maintain insulin independence [115,116]. With each transplant requiring islets from two to three donor pancreata and patients needing subsequent transplantations, a large burden is placed on the already limited donor availability, preventing the procedure from being accepted as a clinical standard and reaching the greater population.

Once isolated from their natural location within the pancreas, islets exhibit diminished survival and function, both *in vitro* studies and soon after transplantation [80,82-84]. Within the pancreas, islets are immersed with their native blood supply. After isolation, diffusion becomes the primary means of oxygen, glucose, and nutrient transport into the core of isolated native islets [82]. Revascularization is essential for islet survival and function after transplantation, but this process can take several days or even weeks to occur [87,117,118]. Diffusion barriers, particularly with respect to oxygen, affect the maintenance of cell viability and performance. The limited diffusion of oxygen into the core of a native islet can lead to reduced function, core cell death, and eventual islet loss. Studies have reported that the size of an islet has a profound effect on its survival and function [80-82,84]. Several studies have demonstrated that small islets (diameter < 125 μm) were superior large islets (diameter > 150 μm) *in vitro*, and showed increased insulin independence when transplanted [80,82-84]. Mathematical modeling of the diffusion of oxygen into the islet core demonstrated the absence of core cell death in islets with a radius less than 50 μm [82]. This was supported by stained images showing apoptotic and necrotic cells within the cores of large islets

in culture as early as 24 hours after isolation. Similarly, the direct glucose diffusion into islets demonstrated that glucose penetrated the core of small islets, while only the outermost layers of cells were penetrated in large islets [82]. The combination of these factors decreases islet survival and overall graft performance.

Engineering optimal islets provides a means to overcome the diffusion barriers affected by islet size limitations. Although dispersing islets into single cells would greatly decrease any diffusion barriers, it is well known that islet cells function better when clustered and are highly dependent on cell-cell communication and the paracrine interactions between the different cell types [67-71,73,74,76,86,119,120]. For several decades, researchers have witnessed the spontaneous reaggregation of dispersed islet cells and have utilized this in reconstructing islet-like tissues [88-93]. Traditionally, islet cells have been incubated in culture medium and allowed to reaggregate into pseudo-islets of a variety of shapes and sizes, often times resulting in pseudo-islets much larger than 200 μm in diameter. The problem with this methodology is the high probability of regeneration of large cell clusters—reintroducing the diffusion barrier, inferior viability, and reduced function. Understanding these size limitations, engineering islets of a small size (less than 125 μm) will help overcome these issues.

Limited studies have been conducted aimed at engineering islets of a defined size, due to complications that may be inherent within their respective techniques. In 2007, islets of a standard size were engineered utilizing the “hanging drop” technique [97]. Although this technique allowed for the creation of uniform islet cell clusters, it required immense manual labor with individually placing drops and the

lacked the ability of scale up for mass production. Even with recent availability of hanging drop plates and the use of automated instrumentation, the technique is still low output and not an ideal solution. A tool that can allow controlled and optimized reaggregation of islet cell clusters can have a considerable impact on islet production for research and transplantation.

In this study, we focused on limiting the diameter during reaggregation of islet cells to that of small islets (less than 125 μm). We engineer optimal islets, or Kanslets, *in vitro* by reaggregating dispersed islet cells on a glass mold containing micro-recesses of a defined geometry. Use of this tool resulted in the production of uniform, reproducible Kanslets. We assessed several characteristics of the Kanslets and compared their function to that of isolated native islets. Finally, we transplanted the engineered Kanslets into diabetic rats and compared the response to matched animals receiving native islets. In summary, we have developed a tool and high output technique for the uniform production of reaggregated islets of an optimal geometry. Kanslets can provide more viable tissue for use in research and transplantation.

MATERIALS AND METHODS

Design and Fabrication of Micromolds

Glass substrates, pre-cut in a disc shape were ordered from Precision Glass and Optics (Santa Ana, CA). The glass substrates were cleaned using acid and base piranha solutions and dried at 200°C to ensure the surface was free of moisture. Glass micromolds were fabricated through a multistep process that included thin-

film deposition, photolithography, and wet etching techniques. Briefly, one surface of the glass substrates was sputtered with a layer of chromium (Lesker Thin Film Deposition System). Positive photoresist (AZ1518) was spin-cast onto the chromium surface and pre-baked at 100°C for 2 minutes. A transparency mask template was created containing the defined geometry and layout of wells to be etched using computer-assisted design (CAD) in AutoCAD software (Autodesk) and high-resolution transparency masks were printed. The photoresist-coated discs were exposed to UV-light through the transparency mask for 4 seconds. Patterned glass was then post-baked at 100°C for 10 minutes and then immersed in developer (AZ 300 MIF Developer) to render the image. The chromium layer was subsequently etched (CR7S Chromium Etchant). Glass was washed with water and dried with nitrogen.

To etch the pattern into the surface of the glass, the disc was wet etched by immersion in a buffered oxide etch (BOE) solution containing a 14:20:66 ratio of HNO_3 to HF to H_20 respectively. A profilometer (Tencor Alphastep 200) was used periodically to measure the etched surface and adjustments were made accordingly. The photoresist and chromium layers were removed to reveal the etched micromold.

To contain the cell culture on top of the glass micromold, a polydimethylsiloxane (PDMS; base and cross-linker; Dow Corning, Midland, MI) ring was cast to surround the glass micromold disc. All micromold components were cleaned and steam sterilized prior to any cell culture use or experimentation.

Islet Isolation, Dispersion, and Reaggregation

Pancreatic islets were isolated and dispersed using our previously published procedures [80,82,104]. Briefly, rats were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine. After the peritoneal cavity was exposed, the pancreatic main duct to the duodenum was clamped, cannulated *in situ* via the common bile duct, and distended with cold collagenase (CLS1, 450 units/ml; Worthington, Lakewood, NJ). After excision, the pancreas was incubated for 20-30 minutes with gentle tumbling in a 37°C incubator. The contents of the tube were washed, passed through a 100 μm mesh screen, and sedimented in a refrigerated centrifuge. The pellet was mixed with Histopaque (density = 1.1085) and centrifuged. The islets, collected from the gradient, were sedimented and washed over a sterile 40 μm mesh cell strainer. Islets were placed into a modified DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic and allowed to recover overnight in an incubator at 37°C and 5% CO_2.

Isolated islets were dispersed into single cell suspensions. Islets were washed twice with calcium- and magnesium-free HBSS (cmf-HBSS) before addition of digestion medium consisting of cmf-HBSS supplemented with 4.8 mM HEPES and papain (5 units/ml; Worthington, Lakewood, NJ). Suspensions were incubated on a rotator at 37°C for 20 minutes. Islets were dispersed by trituration using a pipette until the cell suspension primarily contained single cells. The cells were then washed to remove residual papain and transferred to a customized DMEM:F12-

based, serum-free Kanslet aggregate culture medium. Occasionally, a sample was taken and cell counts and yield were determined using a hemocytometer.

The single cell suspension was then carefully plated onto sterile glass micromolds. Within several minutes, cells began to settle into the recesses of the micromold and were in close proximity to each other allowing cell-cell re-adhesion. Micromolds were incubated for 6-7 days at 37°C and 5% CO_2. Cells reaggregated while limiting the size to no bigger than the recess allowed. Aggregate culture medium was changed every 24 to 48 hours until reaggregated islets were formed. Kanslets were removed by simply washing the micromold several times with culture medium until islets dislodged and were aspirated with a pipette.

Viability

Viability of Kanslets was assayed by placing them in PBS in an Attofluor Chamber. Apoptotic and necrotic fluorophores (Propidium Iodide and YO-PRO-1; Vybrant Apoptosis Assay Kit #4, Molecular Probes, Invitrogen) were added and incubated at room temperature for 15-30 minutes. After staining, confocal images were collected through the center of the islet/Kanslet. Samples were imaged on an Olympus FluoView 300 confocal microscope housed at the Diabetes Research Lab. The area of positively-stained cells was measured and divided by the total islet area to determine the percent viability. All images were collected within 60 minutes of initial exposure to the fluorophores.

A hemocytometer was used to estimate cell number prior to reaggregation of single, dispersed islet cells. Islet diameter was measured using FluoView software (Olympus Corporation) and size distribution was calculated.

Electron Microscopy

Native islets and Kanslets were handpicked and placed in 2% glutaraldehyde in 0.1M sodium cacodylate solution and stored at 4°C until imaging. Samples were rinsed twice in 0.1M sodium cacodylate buffer for 10 minutes prior to post fixation in 1% osmium tetroxide for 1 hour. Rinsing with distilled water was followed by a graded ethanol dehydration (30%, 70%, 80%, 95%, 100%) for 10 minutes each. Samples were rinsed twice in propylene oxide for 15 minutes prior to being infiltrated in a mixture of propylene oxide and Embed 812 resin (Electron Microscopy Sciences, Ft. Washington, PA) overnight. BEEM capsules were used to embed the samples in fresh resin prior to curing overnight in a 70ºC oven. Thin sections, 80nm in diameter, were cut using a Leica UCT ultramicrotome and placed on 300 mesh thin bar grids. Contrast was applied to the sections by adding uranyl acetate followed by Sato's lead stain. Images of islets were captured from random sections using a J.E.O.L JEM 1400 transmission electron microscope.

Glucose Diffusion and Uptake

Glucose diffusion and uptake experiments were completed using a confocal microscope as described previously [82]. A PBS solution containing 0.5 mM 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D glucose (2-NBDG) (Molecular

Probes, Invitrogen) was perifused into a chamber containing Kanslets. Images were captured every 2-5 seconds upon commencing 2-NBDG perifusion and subsequently decreased after several minutes for the remainder of the experiment. The rate of glucose was measured from the time the outer cells became fluorescent to when the core cells became fluorescent. The percentage of 2-NBDG uptake was calculated by measuring the area of fluorescence within the islet that was higher than the background and dividing by the total islet area.

Immunofluorescence and Cellular Composition

Kanslet samples were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). Cellular composition of Kanslets was determined by triple-staining islets for insulin, glucagon, and somatostatin. The following primary antibodies were used: anti-insulin (Abcam or Santa Cruz Biotechnology), anti-glucagon (Abcam) and anti-somatostatin (Abcam). Corresponding secondary antibody conjugates included: Cy2 (Jackson ImmunoResearch Laboratories Inc.), Alexa 647, and Alexa 555 (Molecular Probes, Invitrogen).

Images were obtained using a Nikon C1Si or a C1Plus confocal microscope. Images were acquired at 10X – 100X and analyzed using FluoView or Adobe Photoshop software. To determine the cell composition, the proportion of immuno-labeled cells (alpha-, beta- and delta-cells) was evaluated by counting the number of individual visible cells and dividing them by the total number of cells per islet within the confocal image. Kanslet cellular composition was compared to native islets. Pro-insulin (an insulin precursor molecule) (Abcam) and insulin were stained to

visualize production of new insulin molecules.

Static Insulin Secretion (Static Incubation)

Kanslets were equilibrated overnight in DMEM/F-12 medium containing 5 mM glucose and 10% FBS (low glucose medium). Islets were handpicked using a micropipette and a known quantity of islet equivalents were distributed in 24-well plates. Kanslets were subject to low glucose (5 mM), high glucose (17.5 mM) or high glucose with KCl (30 mM). After 60 minutes of static incubation at 37°C and 5% CO_2, conditioned media samples were collected and frozen at -80°C. The insulin concentration was later quantified using an insulin ELISA kit (Alpco).

Dynamic Insulin Secretion (Perifusion Incubation)

Known quantities of native islets and Kanslets were placed into chambers of a perifusion system at a constant flow rate (250-300 µl/min) at 37°C for 30 minutes. After a 30 minute pre-incubation at a basal glucose level (3 mM), the samples were then subjected to 3 testing conditions during a 3-hour incubation period: 60 minutes of basal condition (3 mM glucose), then 60 minutes of high glucose (20 mM), followed by 60 minutes of basal condition (3 mM glucose). During the perifusion, samples of conditioned medium were collected from the output fraction every 10 minutes. Samples were frozen at -80°C and later analyzed for insulin content using ELISA.

Transplantation

Islet transplantation was completed using our published protocols [80,82]. Diabetes was induced in Sprague Dawley (SD) rats by intraperitoneal injection of streptozotocin (65 mg/kg body weight). Rats were monitored for 1 week to ensure that they were diabetic, with blood glucose levels above 250 mg/dl. Animals were then divided randomly into 3 groups: diabetic control (no transplantation), 1500-2000 IEQ native islet transplantation, or the same volume of Kanslets. On the day of transplantation, rats were anesthetized with 45 mg/kg pentobarbital. The incision site was shaved and cleaned with betadine scrub, and an incision made in the body wall on the left flank. The kidney was exposed through the wound and a small incision made into the kidney capsule. Using a small-bore pipette, native islets or Kanslets were placed under the capsule. The kidney was returned to its original position, the muscle was sutured, and the skin closed with wound clips. Novolog insulin (0.55 units/kg) injections were given twice daily post-transplant until transplants provided the recipient with necessary insulin, which in the case of the Kanslet transplants was within 24 hours of the procedure. Blood glucose levels were checked via blood draw from the tail.

Statistics

Measurements were performed in duplicate or more, with results calculated as averages with standard error (SE). Statistical significance was evaluated using a Student's *t*-test with significance defined as $p < 0.05$.

RESULTS

Size and Morphology

Visualization of the reaggregated Kanslets indicates that they were below 100 μm in diameter, as shown in Figure 4.1A. These islets generally measured less than 100 μm with an average diameter of 40.85 ± 0.39 μm. In contrast, reaggregated islet cells that were not constrained by the micromolds often reaggregated in an unconstrained manner forming mega-islets measuring more than 200 μm in diameter (Figure 4.1B). A size histogram of Kanslets compared to native rat islets is provided in Figure 4.1C.

It is important that the Kanslets return to native islet morphology after formation, including the appearance of microvilli covering the surface of the islet. Figure 4.2A and B compare the islet/Kanslet general morphology illustrating the similarity. Electron micrographs highlight the microvilli that cover the surface of a native islet (Figure 4.2C). The microvilli begin to appear on the surface of the Kanslet within 10 days after reaggregation and removal from the micromold (Figure 4.2D).

Viability

To determine the percentage of viable cells within Kanslets, a viability assay was performed on day 5 (after reaggregation of islet cells). Very few Kanslets exhibited any dead cells, with some exhibiting a few random dead cells. For the most part, dead cells were attached to the surface of the Kanslets, and visibly were not incorporated under the islet capsule (Figure 4.3A). On average, Kanslets

presented viability over 99.7 ± 0.1%, in comparison to 72.4 ± 2.8% viability in the native islets (Figure 4.3B).

Glucose Diffusion and Uptake

To compare the diffusion of glucose within Kanslets to previously published work on native islets, we used a fluorescent glucose analog, 2-NBDG. Islets were exposed to 0.5 mM 2-NBDG though perifusion and monitored under a confocal microscope. Glucose diffused into the intracellular spaces almost as immediately as glucose was apparent in the viewing field. Figure 4.4A shows the lack of glucose diffusion into a native islet compared to full penetration in a Kanslet (Figure 4.4B). In fact, the Kanslet showed superior glucose uptake with diffusion rates that were beyond our ability to resolve with an imaging scan speed of 2 scans/sec. As soon as the glucose-containing media came in contact with the Kanslet, it entered the core. When 2-NBDG fluorescence was analyzed, it was clear that a higher percentage of Kanslet cells were permeated with 2-NBDG compared to native islets (Figure 4.4C).

Cellular Composition

Immunofluorescence staining was conducted to ensure that Kanslets were similar in cellular composition to their native counterpart. Kanslets, triple-stained for insulin, glucagon, and somatostatin, indicate that the three major endocrine cell types were present in our engineered constructs (Figure 4.5A). The distribution of these cell types across the islet appears to be in a random order. In general the composition was not radically different from native islets, even though Kanslets

consisted of a significantly larger portion of beta-cells compared to native islets (Figure 4.5B).

The production of new insulin molecules was identified by staining for the insulin precursor molecule, proinsulin. The presence of proinsulin in islets indicates the synthesis of new insulin molecules (Figure 4.6A). Kanslets also demonstrated the presence of proinsulin levels even 7 days after reaggregation in a pattern similar to native islets (Figure 4.6B).

Glucose-Stimulated Insulin Secretion

In static incubation studies, Kanslets were subjected to low and high glucose concentrations in static culture. Kanslets responded to glucose as expected with increased insulin secretion as the glucose level increased (Figure 4.7A). Kanslets subjected to high glucose resulted in a significant increase in insulin output, double that of the basal level ($p < 0.05$). Exposure to high glucose and potassium (membrane-depolarizing agent) further increased this output to 4 times the insulin secreted at a basal level ($p < 0.001$). This was also significantly higher than output at high glucose alone ($p < 0.001$).

Perifusion experiments were completed to test the insulin secretion over time (dynamic secretion) in response to varying glucose levels. Kanslets were subjected to basal glucose, then high glucose, and again basal glucose over a 3-hour period. Insulin secretion remained stable in response to basal glucose, and resulted with a spike in insulin secretion immediately upon exposure to a high glucose concentration. Insulin secretion more than doubled in response to the presence of

higher glucose (Figure 4.7B). These values were normalized by volume (islet equivalents) and by cell number, and showed little difference between calculation methods [105].

Transplantation

Streptozotocin-induced diabetic rats received transplantation of 1500-2000 IE of either native islets or Kanslets under the kidney capsule. Post-transplant blood glucose values were monitored for an initial 2 weeks following transplant. In both cases, diabetes was reversed within days in rats following transplantation (Figure 4.8). In fact, insulin injections were halted in the rats receiving Kanslets a day earlier than those receiving native islets, because the Kanslets reversed the hyperglycemia faster than the native islets. The rat receiving the Kanslet transplant reached a blood glucose level < 250 mg/dl within 24 hours post-transplant, while the native islet transplantation required 72 hours to return to normal glucose levels. Both groups continued to sustain insulin independence over the 2-week period. In contrast, the control diabetic group failed to reach normoglycemic levels for any of the measurements taken.

DISCUSSION

In this study we utilized a novel method for the uniform production of engineered islets optimized to reduce diffusion barriers. Kanslets provide a feasible alternative to using native islet tissue in research and transplantation. The process minimizes the diffusion barriers necessary for transport of key nutrients including

oxygen and glucose, allowing for increased viability and long-term performance. Uniform, engineered islets were produced by dissociating islets into single cells and utilizing a micromold to optimally reaggregate the cells. This uniformity of size was limited to a maximum diameter of 125 μm, because the critical oxygen concentrations resulting in core cell death above this diameter as previously described [80,82]. By limiting the diameter of this engineered islet tissue, we were able to increase the viability to nearly 100%; a significant improvement over previously published data on native islets [80]. It is likely that the engineering process aids in the elimination of non-viable or dead cells, as islet cells reaggregated to form new clusters.

Further experiments with diffusion of glucose molecules showed a reduction in, or lack of, a glucose diffusion barrier as Kanslets were nearly instantaneously infiltrated with the fluorescent tag. This is in contrast to the published diffusion rate of 2.8 mm/min into native large islets, indicating an extremely slow penetration [82]. Previous experiments with glucose diffusion into native islets indicated that even after 1-hour of incubation, fluorescent glucose penetrated only the outermost layers of a large islet while diffusing with an increased efficiency into small islets [82]. Our results replicated previously published data, while using a much smaller concentration of labeled glucose (0.5 mM 2-NBDG) [82]. Our results show that the diffusion barrier to glucose was markedly reduced and support the necessity to produce islets less than 100 μm in diameter.

The engineering process allowed for successful production of islets that contained alpha-, beta-, and delta-cells, and was similar in composition to their

native islet counterparts. It is unknown why there was a slight increase in beta-cells in Kanslets and may be due to the engineering process. It is possible that during islet isolation from the pancreas, a portion of the outer islet cells, which are predominantly alpha- and delta-cells, may have been lost. This is not necessarily a negative outcome as this restructured composition may be more ideal compared to native islets for both research and transplantation. Allowing the islet cells to self-aggregate into an islet may allow for cell-cell communication and signaling that leads to an increased incorporation of beta-cells. Given that native islets contain blood vessels, there is a great probability that insulin will be secreted close to a vessel. In the Kanslets, diffusion is the primary method of insulin release into the media. Thus, having an abundance of beta-cells on the surface increases the chances of glucose detection and corresponding insulin secretion. The positive staining for proinsulin indicates that even after the reaggregation engineering process, Kanslets are still capable of producing new insulin molecules.

Given the similarities in islet composition, it is not surprising that Kanslets responded to changes in glucose levels as expected. Kanslets secreted more insulin when exposed to high glucose levels over the basal level. This increase could be witnessed in static and dynamic experimental conditions, with insulin levels returning to normal after high glucose stimulation ceased in dynamic experiments.

Kanslets were able to provide a transplantable solution that matches the effect of native islets. As expected, there was a reduction in blood glucose levels in diabetic rats that received the Kanslet transplant. More notably, these levels were similar to those produced by the native islet transplant group. In this study, rats

were transplanted with approximately the same amount of islet tissue. Although our findings show similar results between treatments, it is uncertain at this time whether Kanslet transplants are more potent or outperform native islets with a lower transplanted tissue volume. Kanslets do provide an alternative solution to conventional islet transplantation. Subsequent studies will be necessary to determine the dosage comparison between native islets and Kanslets, and the marginal mass required of Kanslets required to achieve normoglycemia.

With previous studies validating the superiority of small islets to large islets, there was a shift to preferentially utilizing small islets for both research and transplantation [80,82-84]. However, this preference leaves significant numbers of large islet tissue unused. The driving force behind our process was to recreate small islets from native islet cells and limit islet size during the process. This has implications both in research and in clinical transplants. The use of this highly viable tissue in transplantation may increase overall success rates. In research, the production of uniform islet tissue allows for less error between islet preparations and higher reproducibility of data. Utilizing this technology for research and transplantation makes significant strides toward developing the next generation of treatments for diabetes.

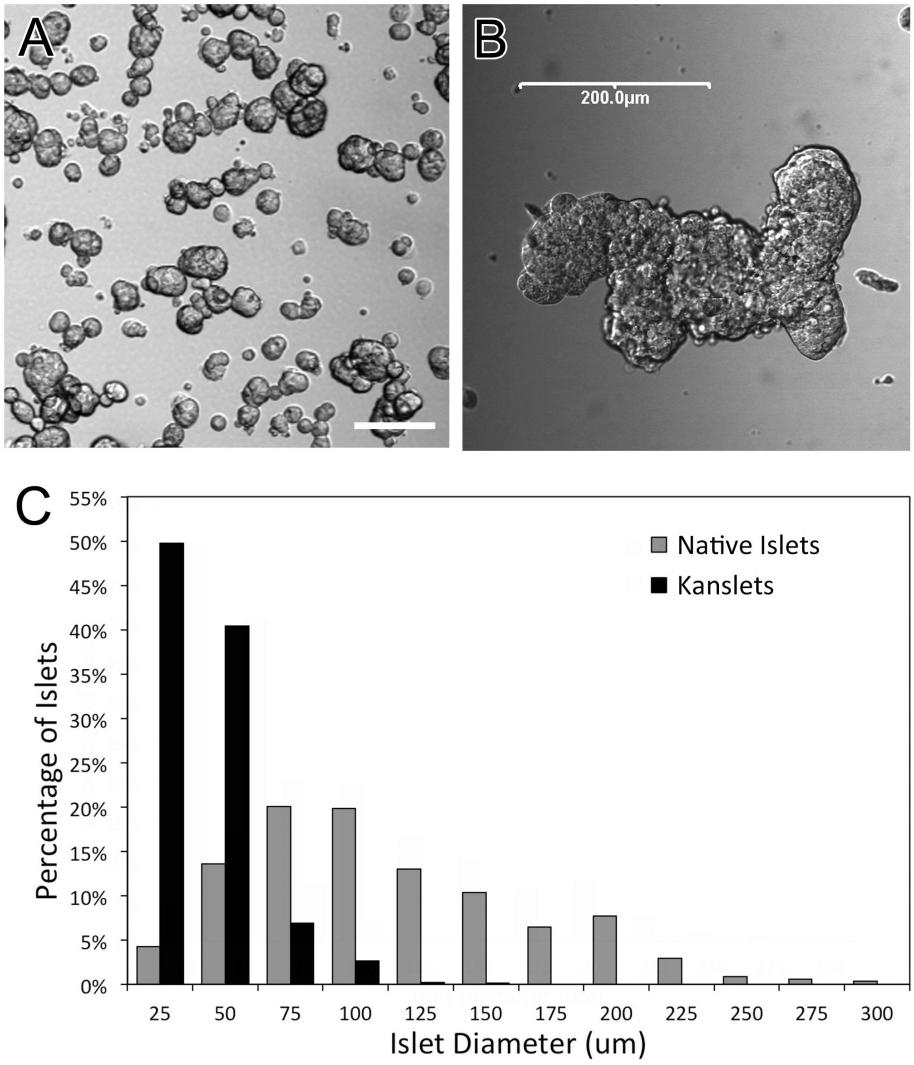


Figure 4.1: Uniformity of Size

(A) The image demonstrates the uniform production of Kanslets below 100 µm. Image was taken 24 hours after removal from the micromold. (Scale bar is 100 µm.) (B) Mega-islets, larger than 100 µm, are a result of unconstrained geometry during islet cell reaggregation. (C) The distribution of native islets and Kanslets according to the diameter. The diameter of Kanslets measured less than 100 µm in comparison to the broad size range of native rat islets. (n = 2323 Kanslets, n = 5974 native islets)

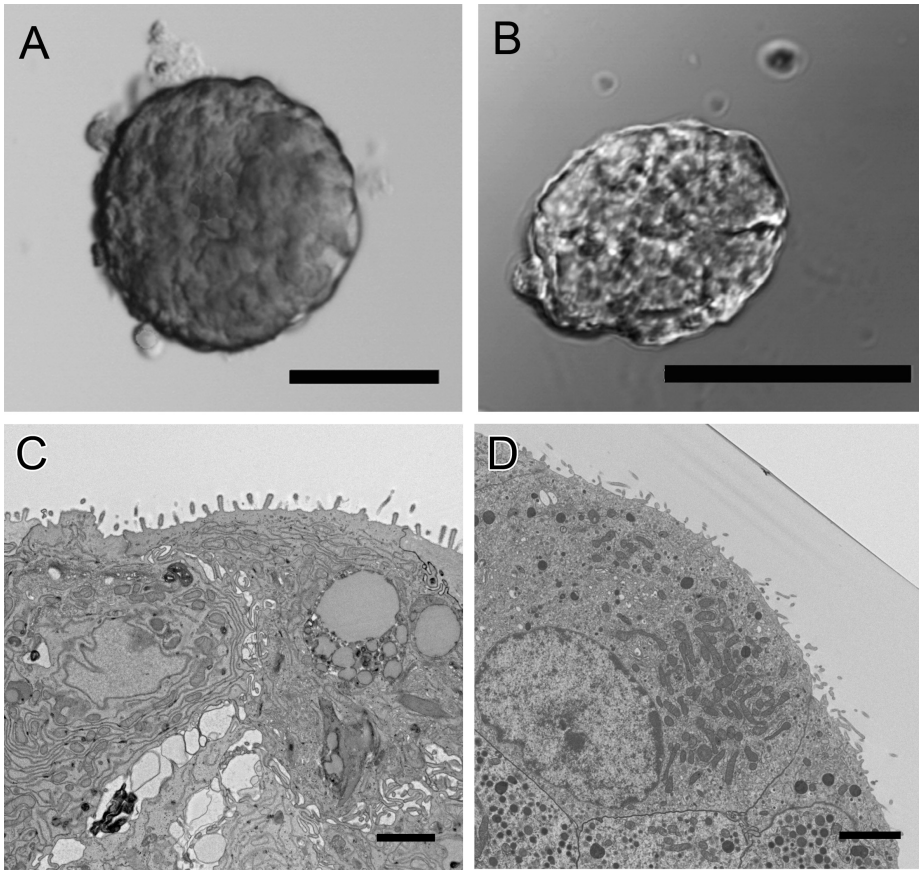


Figure 4.2: Morphological Similarity Between Native Islets and Kanslets

Native small islet (A) and Kanslet (B) have the same general shape and are indistinguishable under light microscopy. (C) Electron micrograph illustrates that the surface of native islets are covered with microvilli. (D) Ten days after Kanslet formation, microvilli immerge on the surface of Kanslets. Scale bars = 50 μm (A,B) and 2 μm (C,D).

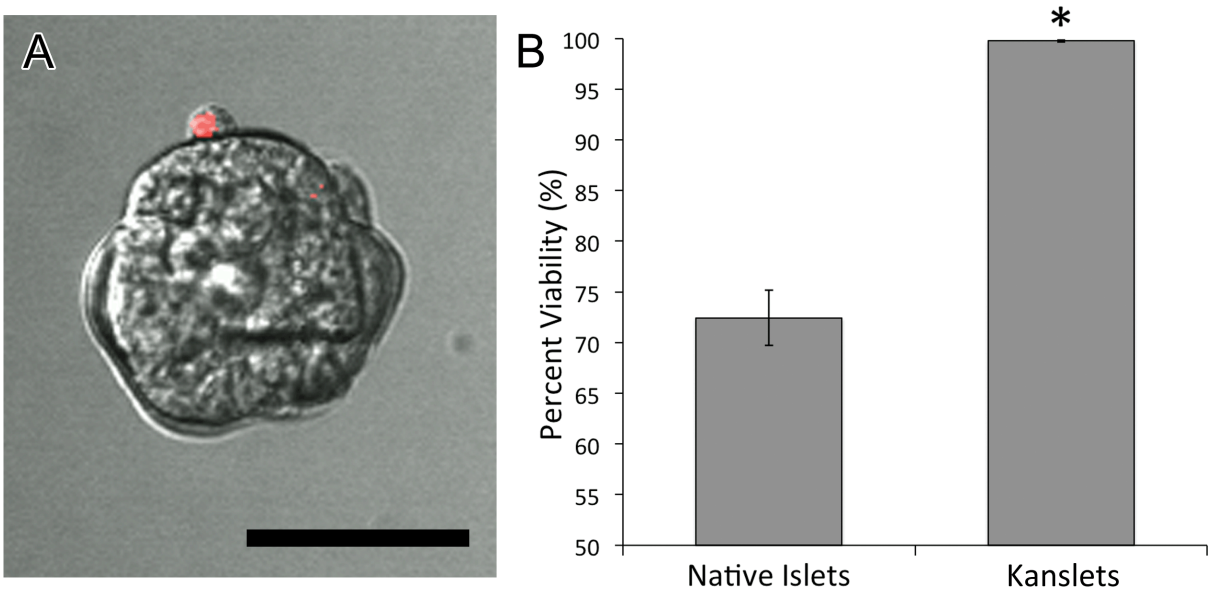


Figure 4.3: Kanslet Viability

(A) Kanslet with one dead cell attached to the side. It is clear to see that the dead cell is outside of the capsule that surrounds the reaggregated islet. (B) Kanslets have significantly greater viability when compared to native islets within 24 hours of isolation or removal from the micromold. All values are expressed as the average ± standard error (n = 510 Kanslets, n = 86 native islets), $p < .001$, * = statistical significant difference from native islets. Standard error of Kanslets is too small to visualize. (Scale bar = 50 µm.)

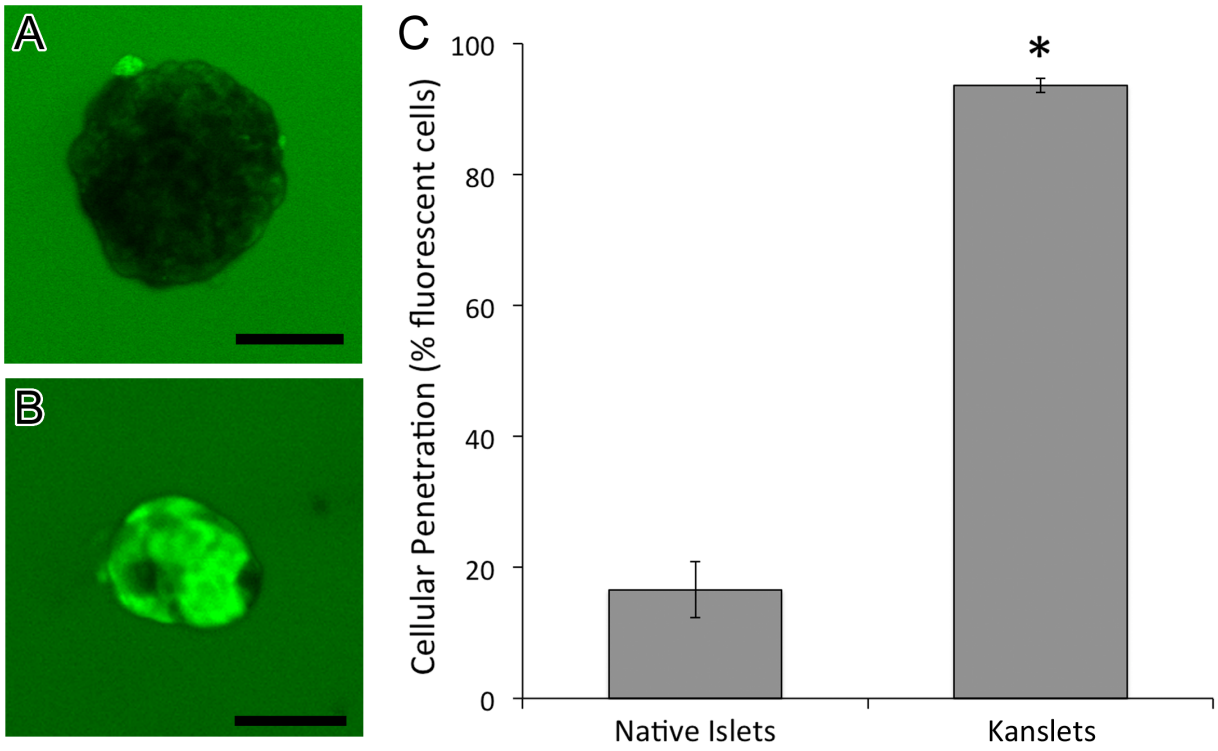


Figure 4.4: Diffusion Barrier of Kanslets

(A) Native islets failed to be permeated with fluorescent glucose (2-NBDG) even in steady state conditions. (B) In contrast, fluorescent glucose was taken up by Kanslets instantaneously upon introduction to the medium. (C) Analysis of the percentage of cells that had 2-NBDG - fluorescence higher than the background shows that cells within Kanslets took up the fluorescent molecule from the media at a significantly higher level than native islets. All values are expressed as the average ± standard error, $p < .001$, n = 45 native islets (average diameter 91 mm), n = 149 Kanslets (average diameter 57 mm), * = statistical significant difference compared to native islets. (Scale bars = 50 μm.)

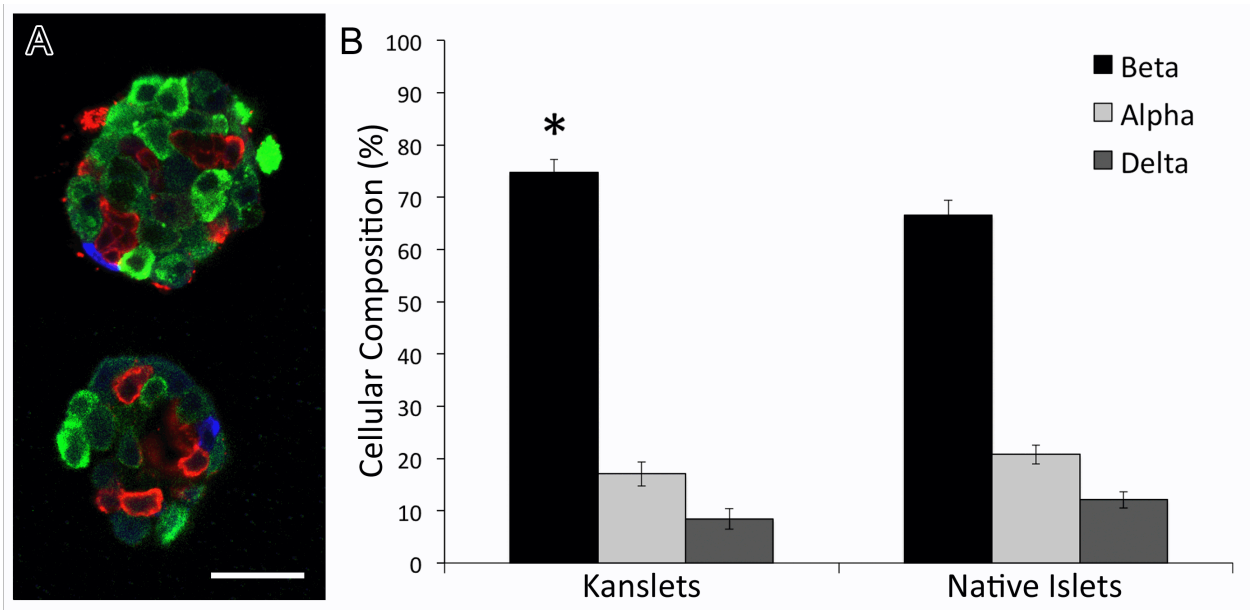


Figure 4.5: Cellular Composition of Kanslets

(A) Triple-stained Kanslets show the presence of three types of islet endocrine cells: beta-cells stained for insulin (green), alpha-cells stained for glucagon (red), and delta-cells stained for somatostatin (blue). (B) The overall islet cell composition was similar between Kanslets and native islets, although there were statistically more beta-cells in the Kanslets than in the native islets ($p < 0.05$). n = 23 Kanslets and 26 native islets (Scale bars = 25 μm.)

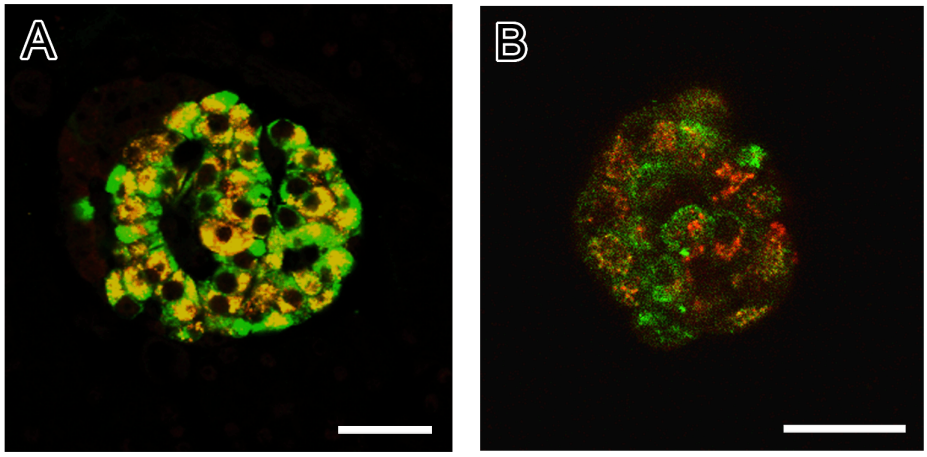


Figure 4.6: Proinsulin Production in Kanslets

Proinsulin (red) is present along with insulin (green) in both isolated native islets (A) and Kanslets (B). (Scale bars = 25 μm.)

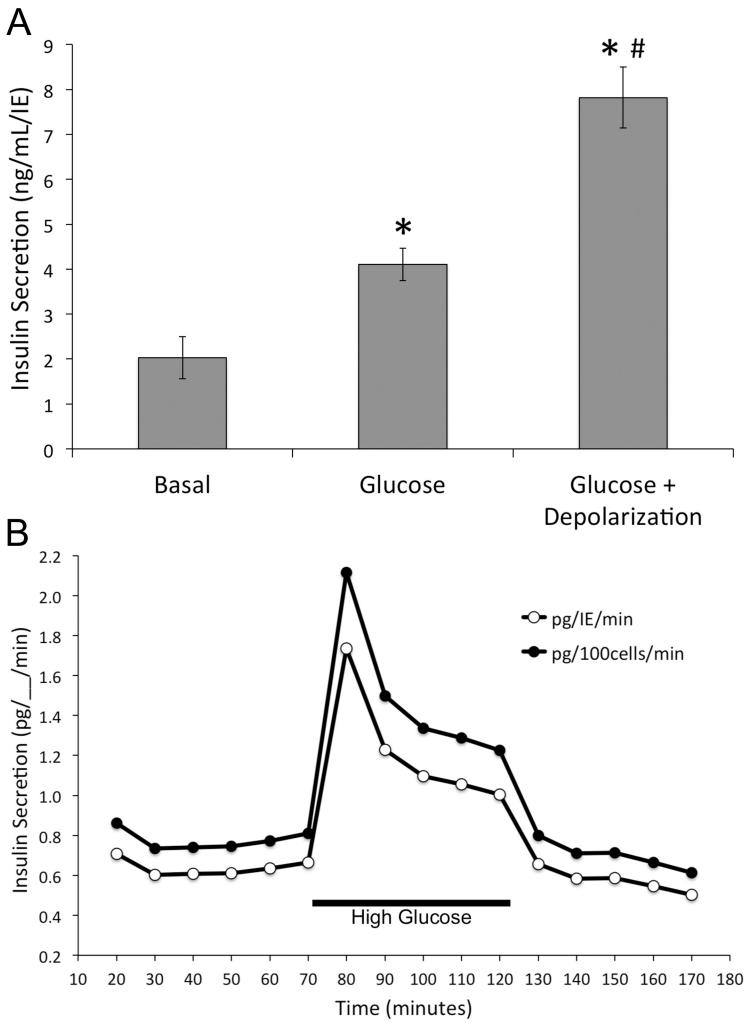


Figure 4.7: Insulin Secretion from Kanslets

(A) Insulin secretion was measured in Kanslets during static incubation in response to varying glucose levels. Kanslets were exposed to low glucose (5 mM), high glucose (17.5 mM), or high glucose with depolarization (30 mM K^+). All values are expressed as the average ± standard error (n = 10 replicates from 4 rats), $p < 0.05$, * = significant increase in insulin secretion when compared to basal glucose stimulation, and # = significant increase in insulin secretion when compared to high glucose stimulation. (B) Kanslets were perifused in low glucose (3 mM), followed by exposure to high glucose (17.5 mM) from 70 - 130 minutes, with a return to low glucose for the remainder of the study. A spike in insulin secretion was evident following the increase in glucose concentration and remained elevated for the duration of high glucose exposure. Results were normalized by islet volume (islet equivalents; open circles) and by cell number (closed circles) (n = 5734 Kanslets).

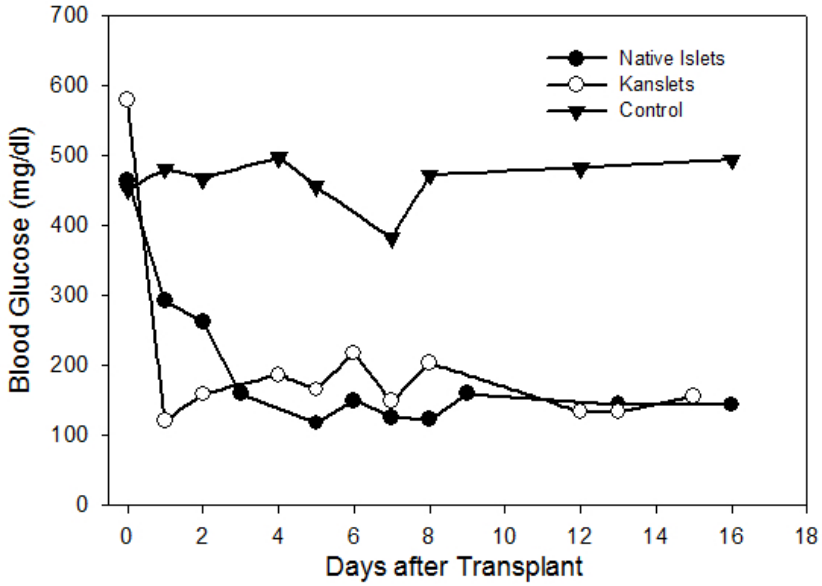


Figure 4.8: Transplantation of Native Islets and Kanslets

Native islets and Kanslets were transplanted into streptozotocin-induced diabetic SD rats (2 rats per group). Rats receiving the same volume of either native islets (filled circles) or Kanslets (open circles) achieved insulin independence and normoglycemia for the 2-week period following transplant with blood glucose levels < 250 mg/dl. Control diabetic rats that did not receive transplants continued to have hyperglycemia throughout the experiment. Day 0 values were obtained on the day of the procedure, but prior to the transplantation.

CHAPTER 5: Exploratory Use of the Micromold Technology as a Platform for Producing 3D Tissue Spheroids for Various Applications Including Drug Discovery and Regenerative Medicine

INTRODUCTION

Millions of individuals are suffering from a wide variety of diseases with cancer, diabetes, and heart disease among the top 10 leading causes of death in the United States. Every year, research efforts are being focused on providing better therapies to improve the quality of life for those suffering from chronic diseases. As these emerging therapies are being developed, there are approaches that can be improved to increase productivity and success. One of the major transitions currently benefiting scientific discovery, is a shift from 2D to 3D cell culture. For decades, researchers have utilized 2D cell culture for research and discovery. With hopes to garner insight into how tissues function within our bodies, the flat morphology of 2D cell monolayers lack significance and can provide misleading results. Basing our understanding of cellular systems and real tissues on the 2D system comes with the underlying assumptions that cell monolayers mimic true nature. Unfortunately, researchers are finding that this is not the case. In seeking a more physiologically relevant solution for *in vitro* testing, researchers are increasingly transitioning from 2D to 3D cell culture.

The 3D cell culture approach is a hot topic right now, with applications in many fields including basic science, drug discovery and development, regenerative medicine, and diagnostics. For instance, tumors exist as spherical structures composed of many cell types working together in a complex fashion to wreak havoc

on a host's body. Cancer researchers have observed significant differences when utilizing 3D tumor spheroids *in vitro* as opposed to 2D cell monolayers [38,41,49-51,121]. In the case of lung cancer therapeutics, a study found that most of the drugs they tested in a 2D monolayer environment had a significantly different result on the same cells when they were in a 3D environment [49]. Using 3D tumor provides researchers with the best solution to understand tumor biology and discover better chemotherapeutics for treatments and cures.

With the large number of 3D cell culture innovations under development, only the most user-friendly, reproducible, cost-effective solutions will be widely accepted by the commercial marketplace. The entire notion behind the use of 3D cell culture is to provide support for cells to behave in a natural manner, mimicking the physiological structure within living systems. A wide assortment of techniques are being utilized to engineer tissues in a scaffold-free manner [45,122]. Spontaneous cell aggregation and self-assembly of dispersed cells, a foundation of many of these techniques, has been a phenomenon extensively studied by researchers [88,95,123-127]. By allowing cells to aggregate, albeit in an *in vitro* environment under controlled conditions, researchers are able to produce tissue-like spheroids.

When developing these techniques certain criteria to consider may include: efficiency, geometric constraint, influence on cell physiology, ease of use, and scalability for commercial applications [125]. Popular methods currently utilized for spheroid formation are spinner cell culture, the hanging drop approach and micromolding. Unfortunately the advantages that each method provides, comes

with its own inherent difficulties that can hinder commercial acceptance [125,128]. For instance, a newly available technology allows the automated production of size-controlled tissues via the hanging drop approach on a specially designed method. With it, comes the disadvantage of low output, evaporation, and small volume medium changes. In general, many of the technologies are being designed for specific applications; however, a universal platform technology for 3D cell culture could provide greater benefit [45].

In developing our approach to 3D spheroid production, we provide a novel platform with the key considerations sought out by the commercial marketplace. This includes providing a user-friendly tool for the efficient, scalable, and high-output production of 3D tissue spheroids. In designing our method, we additionally considered contamination, culture media changes, and amount of manual labor. By incorporating various innovations into the design, we provide a unique platform that is simple to use, applicable for high content screening, allows geometric and size control, and provides a low cost alternative for high-output production. Although initially developed for application to diabetes treatment advancement – diabetes drug development and transplantation, we have expanded the use of this technology in several other applications.

Our technology enables gravity-enforced aggregation and self-assembly of dispersed cells using a micromold providing geometric constraints. The surface contains several thousand conical-shaped recesses per cm^2, specifically tailored for tissue specific requirements or by the researcher. The micromolds were used for the production of islet-like spheroids called Kanslets. In this study, we demonstrate

the ability to use Kanslets for drug testing. Additionally, we discuss how we leveraged our micromold technology for the production of a variety of tissue engineered spheroids.

MATERIALS AND METHODS

Fabrication of Micromolds

Glass micromolds were fabricated through our previously discussed multistep process that included thin-film metal deposition, photolithography, and wet chemical etching. Briefly, glass discs were cleaned using acid and base piranha solutions and dried at 200°C to remove all moisture. Chromium was sputtered onto one surface of the glass substrate (Lesker Thin Film Deposition System). Positive photoresist (AZ1518) was spin-coated onto the chromium surface and prebaked at 100°C for 2 minutes. A transparency mask template, designed using Auto-CAD software (Autodesk), was printed at high-resolution. The photoresist- and metal-coated disc was exposed to UV light through the transparency mask for 4 seconds. Patterned glass was then post-baked at 100°C for 10 minutes and then immersed in developer (AZ 300 MIF) to render the image. The chromium layer was subsequently etched (CR7S Chromium Etchant). After a quick wash and drying, the pattern was etched into the surface using a buffered oxide etch solution containing HNO_3:HF:H_2O at a ratio of 14:20:66, respectively. A profilometer was used periodically to measure the etched surface and adjustments were made accordingly. Finally, the photoresist and chromium layers were removed to reveal the etched glass micromold.

To contain culture medium and cells on top of the micromold, a polydimethylsiloxane (PDMS) ring was molded to surround the glass micromold disc. All components were cleaned and steam sterilized prior to any cell culture use or experimentation.

Islet Isolation and Cell Culture

Pancreatic islets are isolated from Sprague Dawley rats using our previously published protocols [80,82,104]. Briefly, Sprague Dawley rats were anesthetized by an intraperitoneal injection of a mixture of ketamine and xylazine. An incision was made to expose the peritoneal cavity and the main pancreatic duct to the duodenum was clamped. Via the common bile duct, a cannula was thread *in situ* into the pancreas. Cold collagenase (CLS1; Worthington, Lakewood, NJ) was pumped, distending the pancreas. After excision, the pancreas was incubated for 20-30 minutes on a rotator at 37°C. The contents of the tub were washed, passed through a 100 μm mesh screen, and sedimented in a refrigerated centrifuge. The pellet was mixed with Histopaque (density 1.1085) and centrifuged. The islets, collected from the gradient, were sedimented, and washed over a sterile 40 μm mesh cell strainer. Islets were placed into CMRL1066-based media and allowed to recover overnight in an incubator at 37°C and 5% CO_2.

Before engineering islets on the micromold, isolated islets were dispersed into single cell suspensions. Islets were washed twice with calcium- and magnesium-free HBSS (cmf-HBSS). Islets were then incubated on a rotator at 37°C for 20 minutes in cmf-HBSS with 4.8 mM HEPES and papain (5 units/ml;

Worthington, Lakewood, NJ). Islets were dispersed by trituration using a pipette until the cell suspension primarily contained single cells. The cells were then washed to remove residual papain and transferred to DMEM-based or CMRL-based culture medium and kept in suspension for spheroid production.

Cancer Cell Line Culture

Established lung cancer cell lines included small cell lung carcinoma (NCI-H69), epithelial lung carcinoma (A549), and bronchioalveolar carcinoma (NCI-H358). H69, H358 were originally grown in RPMI 1640 media, and A549 was grown in Hams F12K media, both supplemented with 10% fetal bovine serum (FBS) and 1% Anti/Anti. Dr. G. Sitta Sittampalam graciously provided cell suspensions for immediate use in spheroid production.

Bone Marrow Mesenchymal Stem Cells (BMSC) Harvest and Culture

Bone marrow-derived mesenchymal stem cells were harvested from the femurs of exsanguinated Sprague Dawley rats. Legs were completely removed at the hip joint and the skin and foot were removed. The leg bones were then placed in a calcium- and magnesium-free PBS solution for 30 minutes. After removing the muscles, the femur was exposed and detached at the knee. The ends of the femur were carefully cut using a bone cutter or rongeur. Using an 18-20 gauge syringe needle, the medullary cavity was flushed with PBS and expelled bone marrow was collected in a microcentrifuge tube. The bone marrow was subsequently triturated to disperse large tissue clumps into a more uniform solution. BMSCs were isolated

using plastic adherence culture. The bone marrow suspension was plated on tissue-treated flasks with Alpha-MEM containing 10% FBS and 1% Anti/Anti. Culture medium was changed carefully every 24 hours for the first 72 hours, then every 3-4 days until they reached 70-90% confluence. Aspirated culture medium was discarded, leaving behind BMSCs that had adhered to the flask surface.

When ready for passaging or spheroid production, cells were trypsinized from the flask surface using 0.05% trypsin/EGTA (Gibco) and incubation at 37°C for 5-10 minutes. The cell suspension was collected from the flask and neutralized by adding complete culture medium (containing FBS). The suspension was then pelleted and resuspended in fresh culture medium. If passaged, cells were resuspended in AlphaMEM-based media, split into two, and plated into new flasks. For spheroid production, cells were resuspended in DMEM-based media. Both media contained 10% FBS and 1% Anti/Anti.

Human Umbilical Cord Mesenchymal Stem Cell (HUMSC) Culture

Cryopreserved HUMSCs were graciously provided by Dr. Omar Aljitawi. Tubes were thawed at room temperature and transferred into flasks containing low glucose (5.5 mM) DMEM with 20% FBS + 1% Anti/Anti. After 24 hours and cell adherence, culture media was changed to low glucose DMEM with 10% FBS and 1% anti/anti. Medium was changed every 48 hours until confluent. Cells were trypsinized using the previously described technique for passaging or spheroid production. Cells were suspended in DMEM-based medium for spheroid production.

Tissue Spheroid Production

To produce tissue spheroids, a cell suspension was loaded onto the micromold surface with appropriate culture medium. In the case of hybrid islets with stem cells, cells were mixed together before loading onto micromolds. The micromolds were incubated at 37°C and 5% CO_2. Culture medium was changed every 24-48 hours. After 5-10 days (depending on tissue), 3D tissue spheroids were removed from the micromolds by washing the mold several times with culture media and collecting the suspension. The spheroid containing suspension was then plated in large petri dishes and maintained in an incubator (37°C, 5% CO_2) for experiments.

Kanslet Drug Testing

Drug dose-response studies were completed on Kanslets using Byetta (exenatide; Amylin Pharmaceuticals) and another known stimulant of insulin secretion. Drugs were prepared in high glucose DMEM:F12-based culture media containing 17.5 mM and 11.25 mM glucose, respectively. Serial dilutions were then completed to prepare a variety of doses, with no addition of drug as a control. Kanslets were pre-incubated overnight in low glucose (5 mM) DMEM:F12-based culture media. Known islet equivalencies of Kanslets were dispensed into 24-well plates containing the various drug doses. Following a 60 minute static incubation at 37°C and 5% CO_2, conditioned media samples were collected and frozen at -80°C for ELISA. The insulin concentration was later quantified using an insulin ELISA kit

(Alpco). Runs were completed in duplicates and calculated as an average response ± standard error. Dose-response curves were generated using Microsoft Excel.

Microscopy and Viability

Images were obtained using a Nikon inverted light microscope with attached digital camera or an Olympus FluoView 300 confocal microscope housed at the Diabetes Research Laboratory at the University of Kansas Medical Center. Images were acquired at 10-100X and analyzed using Olympus FluoView or Lumenera Infinity Software.

Tissue spheroids were assayed for viability using apoptotic and necrotic fluorophores (propidium iodide and YO-PRO-1; Vybrant Apoptosis Assay Kit #4, Molecular Probes, Invitrogen). Spheroids were incubated at room temperature in an Attofluor Chamber in media or PBS containing the fluorophores for 15 minutes. After staining, spheroids were imaged using the Olympus confocal microscope.

RESULTS & DISCUSSION

The use of 3D tissue spheroids provides an *in vitro* model that better mimics *in vivo* tissue function and behavior. Our goal in this study was to demonstrate the ability to leverage the micromold technology for 3D culture using a variety of cell types. In previous studies we have produced and characterized islet-like tissue spheroids, or Kanslets. In this study, using a similar technique, we produced cancer spheroids, stem cell spheroids, and hybrid islets (containing both stem cells and

islet cells). Additionally, we conducted preliminary studies to demonstrate the ability to use Kanslets for *in vitro* drug studies.

Engineering Tumor Models

Utilizing several lung cancer cell lines, we were able to produced monotypic spheroids. Cancer spheroids can be made monotypic (composed of a single cell type) or heterotypic (multiple cell types). Initially, our experiments yielded little success with cancer cells adhering directly to the micromold surface. After experimenting with various culture media conditions and techniques, we were able to aggregate cancer cells into spheroids. Cancer spheroids were produced using a lung cancer cell line, NCI-H358 (Figure 5.1). These tumor spheroids began as small cluster, but over the next several day aggregation period grew into large clusters. Compared to the large cluster sizes discussed in the literature (diameter > 300 μm), these clusters measured well below 300 μm and remained highly viable [48,129].

Through the experiments, we found that although cancer spheroids may initially aggregate less than 100 μm in diameter, after removal from the molds, they expanded during cell culture. It appeared that cancer cells continued to replicate during and after aggregate formation. Future studies are necessary to determine the appropriate sizes that will work for drug testing and experimentation. Additionally, cancerous tumors often contain hypoxic or necrotic cores that release a lot of chemical factors inducing local cell death [38,129]. As we move forward with this approach, our next step will aim to produce organotypic cancer spheroids. These

spheroids will be heterotypic, containing multiple cell types, and should increase physiological relevance.

Engineering Stem Cell Spheroids

Stem cell use in research and emerging technologies is becoming increasingly popular [130]. Stem cells are generally unspecialized cells that maintain their innate ability to self-renew or proliferate repeatedly, but can differentiate into a variety of specialized cells under the appropriate environmental conditions, becoming the building blocks of tissues [130,131]. Stem cells can come from a variety of sources including embryonic stem cells, induced pluripotent stem cells, or adult stem cells, just to name a few. They can be harvested from a patient's own body (autologous stem cells) or from another human donor (allogeneic). Stem cells are already being used clinically for bone marrow transplants. The production of stem cell spheroids could have a profound impact in a variety of fields. For example, it could provide a new tissue source for regenerative medicine, or be used as a platform to discover new drugs that aide tissue healing and growth.

We produced two types of stem cell spheroids using bone marrow-derived mesenchymal stem cells (BMSCs) and human umbilical cord mesenchymal stem cells (HUMSCs) (Figure 5.2). Our studies with stem cells have been limited so far and have simply been conducted to demonstrate stem cell spheroid production using the micromolds. As we move forward, studies will include the use of stem cells as a drug testing platform, and in various regenerative technologies and therapies.

Engineering Hybrid Islets: Mixing Islet Cells Together with Stem Cells

In diabetes research, the limited supply of donor pancreata places a large strain on advancing islet transplantation as a cure for diabetes. Increasingly more and more, researchers are looking for alternative tissue sources. A recent review discusses the potential for utilizing stem cells as an alternative source [132].

One option is to convert stem cells into islet-like cells. Currently, the stem cell differentiation requires a complex protocol of growth factors, small molecules, and other culture conditions to generate insulin-producing islet-like cells [133-138]. Researchers may find difficulty in mimicry of islet behavior without an islet-like 3D structure or the inclusion of the other islet cell types (alpha cells, delta cells, etc.) that are naturally present within native islets [71-73,138]. Certainly, this work is on track toward providing a transplantable alternative, however it may come with unintended situations. As mentioned previously, stem cells maintain the innate ability to replicate in number. Although the proliferation pathways may be stopped during differentiation into islet-like cells, this may not always be the case. Engineered cell lines can continue to maintain some oncogenic characteristics [66]. In any case, transplantation of transformed stem cell can have cancerous outcomes.

Instead of transforming stem cells, another option may be co-transplanting or co-culturing them together with islets. BMSCs provide beneficial effects for islet co-culture and co-transplantation including protection against cell damage increasing islet survival and function, natural transdifferentiation into islet-like tissue, and supporting angiogenesis [132,139-142]. More specifically, co-transplantation of islets surrounded by autologous stem cells decreases the

occurrence and chances for graft-versus-host disease and other immune reactions [132,143,144]. It has been recently discovered that the same may hold true for co-transplantation with allogeneic stem cells [145].

As opposed to simply surrounding a native islet with stem cells, engineering islets that include BMSCs could provide another method for the incorporation of stem cells into islet transplantation settings. We combined BMSCs and islet cells together in their dissociated state, and created a hybrid islet using the micromold (Figure 5.3). In a quick viability assessment it appeared that co-aggregation may be beneficial for islet survival (Figure 5.3B). Studies are to be conducted to characterize these hybrid islets examining properties such as stem cell transdifferentiation, functionality, and transplantation outcomes. Hybrid islets could potentially increase the amount of source tissue, while providing a number of benefits from the stem cells alone. Ultimately, this could be an approach, or a solution, to conquer the limited availability of donor tissue for islet transplants, and allow the transplant recipient to live a disease-free life without immunosuppression.

Production of Cell Spheroids for Drug Discovery

In producing these spheroids using a variety of cell types, they could be used for drug screening, *in vitro* drug studies, and a number of other critical experiments involved in drug development. The key in developing a suitable 3D cell spheroid technology is the inclusion of characteristics such as size uniformity, normal and uniform drug responses, predictable and reliable ED50s, organotypic structure, low diffusion barrier, high viability, high commercial yield, and is compatible with

standard industry equipment. Granted we are in the process of conducting drug studies using Kanslets, our preliminary data suggest that we are moving in the right direction.

Kanslets were subject to two different drugs known to produce a stimulatory effect on insulin-secretion from islets. Exenatide (exendin-4), a glucagon-like peptide-1 (GLP1) analogue, is an injectable drug used to enhance glucose-stimulated insulin-secretion. As predicted, in the presence of exenatide and high glucose, Kanslets showed increased insulin secretion with increasing drug dose (Figure 5.4). The range of doses tested was very small and future studies will utilize a log scale range of exenatide doses for full testing. This stimulatory effect was also witnessed with another unnamed drug known to enhance insulin secretion (Figure 5.5). In ongoing studies, we are experimenting with the effect of various known stimulatory and inhibitory compounds on Kanslets. These studies are being conducted in collaboration with a large pharmaceutical company, and the results cannot be disclosed at this time.

CONCLUSION

This study provides a glimpse into the ability of our micromold technology to produce a variety of tissue spheroids. Although we have only scratched the surface with diabetes, cancer, and stem cells, there are many other options for future work. This innovative solution brings physiological relevance to traditional cell culture and bridges the gap between *in vitro* experimentation and clinical care. Building a suitable system to produce 3D tissue spheroids impacts drug discovery,

regenerative medicine, and more. It revolutionizes how we discover the next generation of pharmaceuticals and therapies.

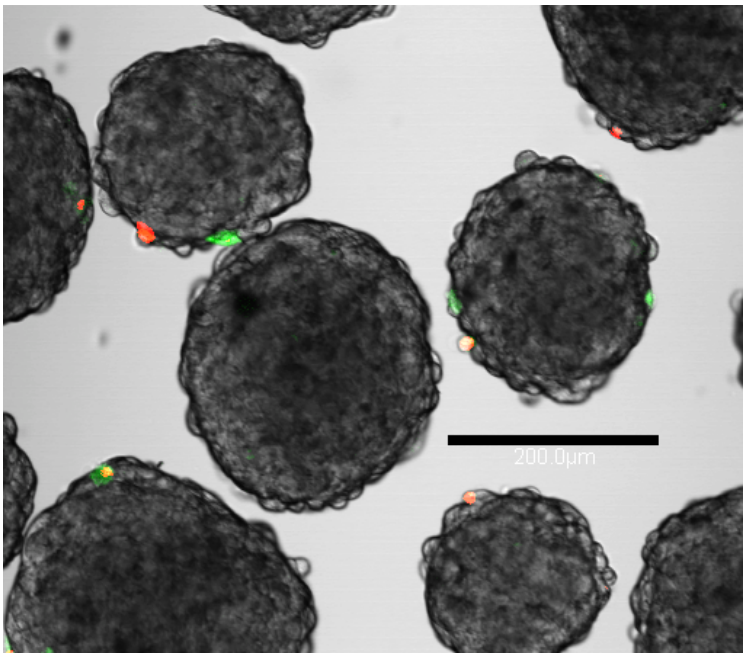


Figure 5.1: Cancer Cell Spheroids

Cancer spheroids produced using NCI-H358, a lung cancer cell line. These spheroids were imaged 4 days after removal from the micromolds (12 days after initial seeding on micromolds). These spheroids remained below 300 µm and maintained a high viability as seen by the lack of fluorescent staining. Red indicates necrotic cells and green indicates apoptotic cells. Scale bar = 200 µm.

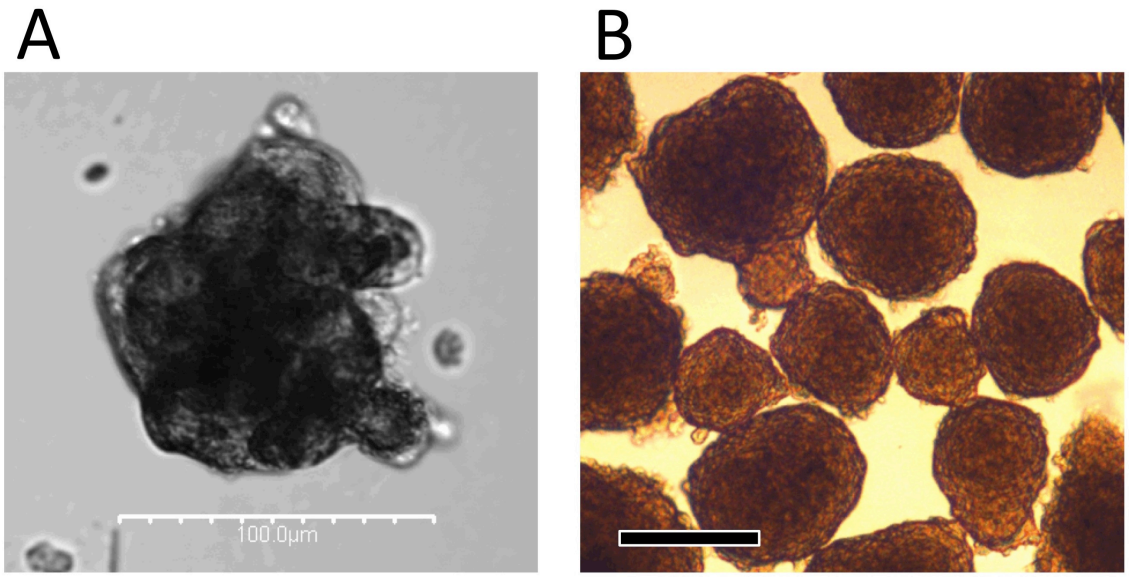


Figure 5.2: Stem Cell Spheroids

Stem cell spheroids composed of (A) bone marrow-derived mesenchymal stem cells (BMSCs) and (B) human umbilical cord mesenchymal stem cells (HUMSCs). Scale bar: 100 µm and 200 µm, respectively.

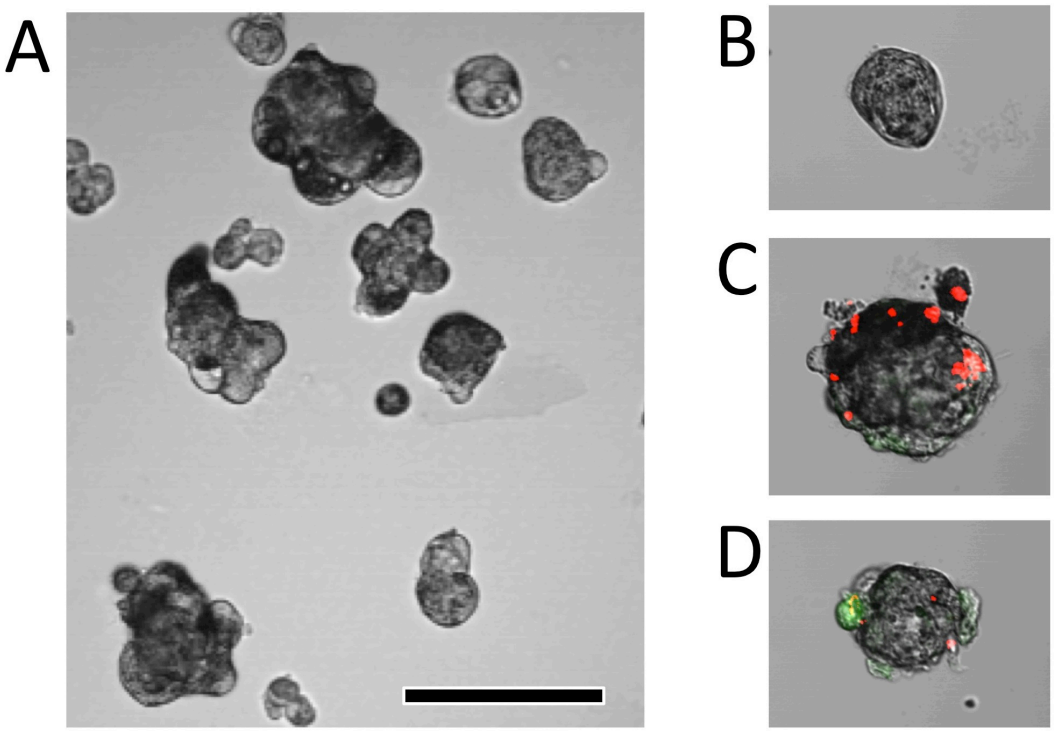


Figure 5.3: Hybrid Islet Spheroids

These hybrid islets are composed of islet cells and BMSCs (A-D). Fluorescent staining for viability indicates the presence of very few dead cells (B-D). Red indicates necrotic cells and green indicates apoptotic cells. Scale bar = 100 μm.

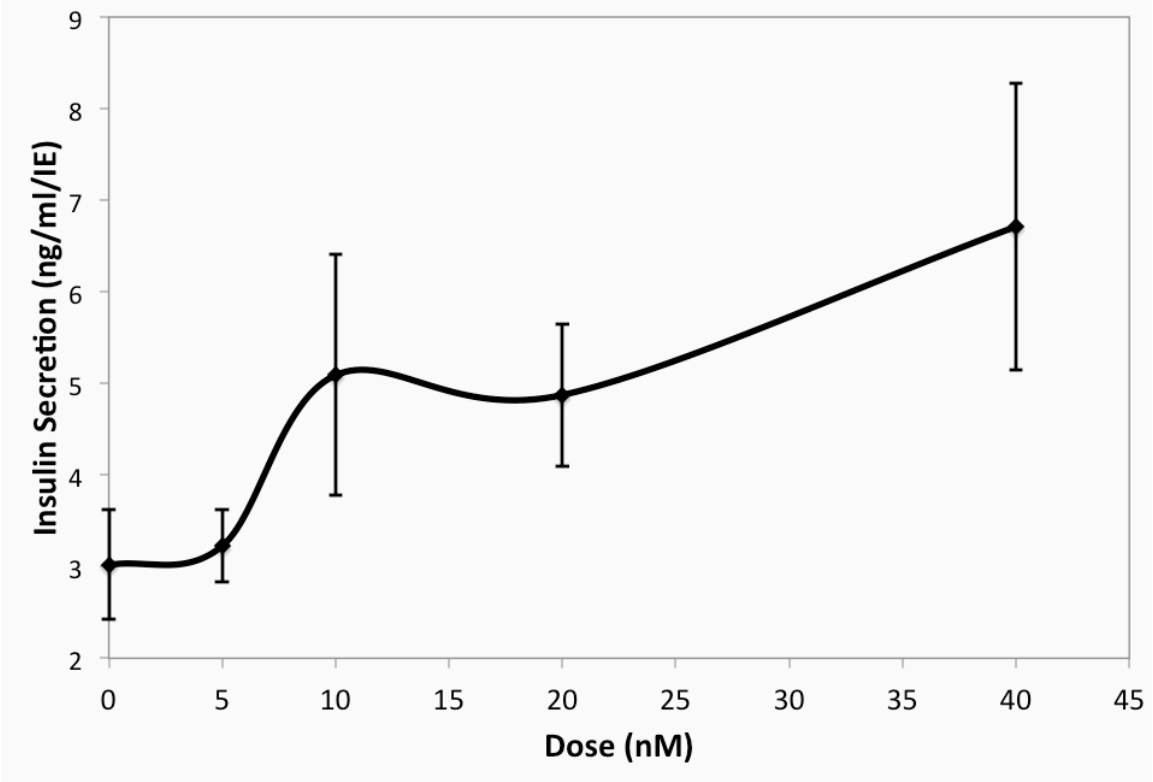


Figure 5.4: Exenatide Enhanced Insulin Secretion

Kanslets were subject to various doses of exenatide. As dose increased, Kanslets responded with an increase in insulin secretion. Error bars indicate standard error.

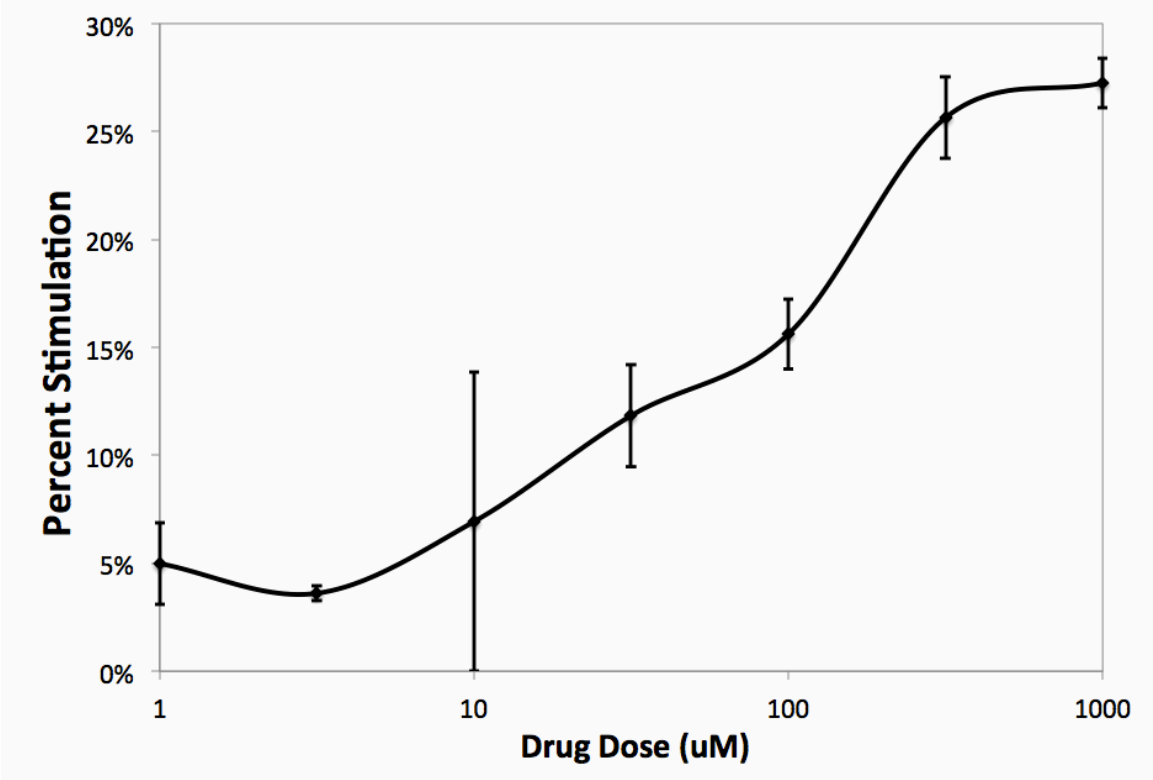


Figure 5.5: Dose-Response Curve of a Stimulatory Drug on Insulin Secretion

Kanslets were subject to a drug known to stimulate insulin secretion. Again, as drug dose increased, Kanslets responded with an increase in insulin secretion. Error bars indicate standard error.

CHAPTER 6: Conclusion

With costs for drug discovery and development rising, and patent expiration dates quickly approaching, there is an ever-growing need for technologies that bridge the gap between *in vitro* experimentation and *in vivo use.* The cells and tissues that compose organs within our bodies are arranged in complex 3D structures. It is less than ideal to use traditional 2D cell culture to garner insight and make inferences about topics such as disease states, drug action mechanisms, or *in vivo* cell behavior. Researchers have embraced the transition from flat, cell monolayer cultures to 3D cell culture supporting structure, growth, and function.

Beta-cell monolayers are the norm in diabetes research. Unfortunately, these transformed cell lines lack proper significance and function, especially without the other islet cell types that are present naturally. Inherent size-dependent limitations within isolated, native islets prevent them from use as a viable alternative. This calls upon engineering an optimal islet-like spheroid.

With many fields only beginning to make a transition to 3D cell culture in the last decade, new innovations in cell culture technology are gaining popularity. Unfortunately, these technologies and techniques come with inherent caveats. Problems such as scalability, reliability, ease of use, and cost, hamper commercial acceptance.

The primary goal within this dissertation was to develop a tool for optimally engineering 3D cell clusters. I designed a micromold that enabled cell aggregation while maintaining an optimal geometry. For islet cell reaggregation, the micromold optimized size by limiting tissue aggregation within a specific size range. These

islet-like spheroids, or Kanslets, mimicked the structure and function of native islets. *In vitro*, Kanslets were highly viable and maintained glucose sensitivity, insulin secretory response, islet morphology and structure. In addition to the superior viability, the reengineering process allowed for increased diffusion rates. A preliminary Kanslet transplant into diabetic rats reversed diabetes, indicating similar outcomes as native islet transplants. More animal studies are necessary to demonstrate the feasibility of Kanslets as a viable alternative for islet transplantation.

The primary goal of using Kanslets as opposed to native islets is to provide a tissue solution that reduces the number of donors necessary for a single recipient. With the high demand for donor tissue, using multiple donors to treat a single recipient limits the availability of islet transplants. The superior viability of Kanslets can potentially alter the short-term rejection of donor cells. The decreased diffusion barrier could allow increased revascularization of the Kanslets, providing for better functionality. Immunogenicity still remains a long-term concern. In current rodent transplantation studies, the donor and recipient are of the same strain, having very similar genetic compositions. Using a donor of a different strain compared to the recipient may offer a better model to investigate immunogenicity. One possible solution within the micromold technology, may be the incorporation of stem cells to protect Kanslets or hybrid islets from graft-vs-host disease. Further characterization of hybrid islets, both *in vitro* and *in vivo* will be necessary.

In terms of use in the commercial setting, Kanslets provided a better alternative for high-throughput screening. Their similarity to native islets offers 3D

spheroids that could possibly be used for drug testing, and their smaller size range may offer better dispensing using the standard industry equipment. An in-depth analysis is necessary to provide further support for using Kanslets as a primary and secondary screening tool for drug discovery.

Being the primary tissue model in this research, Kanslets are the furthest along, and could possibly become the first commercializable product to come out of this work. This technology in not only applicable for diabetes research. I also produced 3D cell spheroids using cancer cells and stem cells. Although these experiments were preliminary, they explored the suitability of the micromolds for 3D cell culture. With industry looking for simple, highly effective technologies for 3D cell culture, the innovations within this technology enable applications in drug discovery, regenerative medicine, and a variety of other fields.

Giving that 3D cell culture is just beginning to catch on, as we think about the future use of this platform, we realize that we have only scratched the surface. In addition to cancer cells and stem cell, the micromolding process could work for a variety of other cell types. For example, chondrogenic and osteogenic cells could be optimally aggregated, providing tissue spheroid building blocks for a variety of regenerative approaches. Using a variety of cell types, we can build organotypic spheroids with applications for drug testing and toxicology, autologous cancer cell spheroids for personalized medicine and diagnostics, and stem cell aggregates for regenerative medicine approaches. This technology can benefit the tissue engineering field and significantly impact the discovery and development of next generation technologies.

Appendix A: Tissue Culture Medium.

During the process of engineering islets, it is important to provide appropriate tissue culture medium for optimal reaggregation. Two formulations of tissue culture medium was developed for use during Kanslet production: a drug testing culture medium and a tissue transplantation culture medium. The prepared culture mediums are either DMEM:F12-based or CMRL 1066-based.

Tissue culture media are available with an assortment of glucose concentration varying from 0 to 55 mM. Specific concentrations may be chose to mimic the various stages of blood glucose associated with diabetes. These glucose concentrations are: 1 – 5.5 mM (low/normal/basal), approaching 10 mM (pre-diabetic), and above 10 mM (high/diabetic). Commercially available DMEM:F12 contains 17.5 mM glucose. Engineering islets at this high glucose level is analogous to culturing at diabetic blood glucose levels. To eliminate the effects of this high glucose condition, a low glucose DMEM:F12 was specifically formulated containing 5 mM glucose. A 50:50 mix of no glucose DMEM (0 mM glucose) is mixed with Ham's F12 (10 mM glucose) to produce a DMEM:F12 combination containing 5 mM glucose, while maintaining similar concentrations of the other additional components contained in standard DMEM:F12. Commercially available CMRL 1066 typically contains 5.56 mM glucose and was left unchanged. The following culture media are prepared using standard CMRL 1066 or our low glucose DMEM:F12 formulation (Table A.1).

Table A.1: Tissue culture medium formulations.

Drug Testing Media	Transplantation Media
DMEM:F12 or CMRL 1066 Fetal Bovine Serum (FBS) Antibiotic/Antimycotic L-glutamine (for CMRL)	DMEM:F12 or CMRL 1066 Insulin-Transferrin-Selenium (ITS) Bovine Serum Albumin (BSA) Epidermal Growth Factor (EGF) Exendin-4 HEPES Buffer Antibiotic/Antimycotic L-glutamine (for CMRL)

The drug testing media is a traditional standard media formulation and is similar to that which we have used in previously publications [56,82]. Our drug testing media contains 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic. Specifically for our CMRL formulation, we included 2 mM L-glutamine in cases where CMRL 1066 was purchased without glutamine.

For the purposes of islet transplantation, native islets and Kanslets should not come in contact with any bovine-derived products. This minimizes the risk of transmitting bovine spongiform encephalopathy, better known as mad-cow disease. Our transplantation media is produced as a serum-free media containing no bovine or other animal products. Serum is very complex, primarily composed of serum albumin and growth factors. Additionally, it can contain proteins, hormones, amino acids, and other components; however, the effects on cell culture are largely undetermined. The components in our transplantation media compensates for components typically present in FBS. Bovine serum albumin composes a major portion of FBS.

Our transplantation media is a modified version based on a formulation provided by Kikugawa, et al. [146]. Our transplantation media uses either DMEM:F12 or CMRL 1066 supplemented with BSA, insulin-transferrin-selenium (ITS), epidermal growth factor (EGF), exendin-4, HEPES, antibiotic/antimycotic. The addition of BSA, ITS, EGF, exendin-4 is largely based on the formulation provided by Kikugawa, et al. [146].

In developing this medium we experimented with the addition of nicotinamide and extra calcium. Nicotinamide is an amide of nicotinic acid and is a part of the vitamin B group. Many research groups include nicotinamide in the formulation of media used for islet cell culture to aid islet isolation, protect against DNA damage, and maintain glucose-stimulated insulin-secretion [133,137,146-148]. Nicotinamide, also known as niacinamide is found as a component of DMEM:F12 and CMRL 1066. A recent study comparing various media formulations containing nicotinamide found that the standard commercial concentration was sufficient and the supplementation of additional nicotinamide (10 mM) showed no beneficial effects [149]. Hence, our formulation does not include additional supplementation of nicotinamide.

Initially, we experimented with addition of extra calcium (26 mM) to our culture media to increase cell-to-cell adhesion. Cells can naturally maintain calcium concentrations without any toxic effects. The removal of calcium and magnesium from media is used during islet cell dissociation, as these metals are important in for many adhesion molecules. Media containing calcium and magnesium is used to stop dissociation and the effects of papain digestion. When cells are subject to a papain

digestion, the cell membrane can become permeable allowing free flow of calcium ions. The extra calcium can have a toxic effect on cells. Immediately upon reintroduction of calcium to the media, the increased influx of calcium into the cells can lead to toxic calcium levels well above the physiological range. Standard media formulations contain calcium in a low millimolar range (0 – 3 mM). In our experience with additional calcium, we found that reaggregation appeared to be limited and this may be attributed to the possible toxic effects and cell death.

BIBLIOGRAPHY

1. Wilson, J.F. Alterations in processes and priorities needed for new drug development. *Ann. Intern. Med.* **145**, 793-796, (2006).

2. Padhy, B. & Gupta, Y. Drug repositioning: Re-investigating existing drugs for new therapeutic indications. *J. Postgrad. Med.* **57**, 153-160, (2011).

3. Pharmaceutical Research and Manufacturers of America. Pharmaceutical industry profile 2011. 1-60, (Pharmaceutical Research and Manufacturers of America, Washington, D.C., 2011).

4. Paul, S.M., Mytelka, D.S., Dunwiddie, C.T., Persinger, C.C., Munos, B.H., Lindborg, S.R. & Schacht, A.L. How to improve r&d productivity: The pharmaceutical industry's grand challenge. *Nat. Rev. Drug Discov.* **9**, 203-214, (2010).

5. Bleicher, K.H., Böhm, H.-J., Müller, K. & Alanine, A.I. A guide to drug discovery: Hit and lead generation: Beyond high-throughput screening. *Nat. Rev. Drug Discov.* **2**, 369-378, (2003).

6. Giersiefen, H., Hilgenfeld, R. & Hillisch, A. Modern methods of drug discovery: An introduction, in *Modern methods of drug discovery*. (eds A. Hillisch & R. Hilgenfeld) 1-18 (EXS, 2003).

7. Munos, B. Lessons from 60 years of pharmaceutical innovation. *Nat. Rev. Drug Discov.* **8**, 959-968, (2009).

8. Kola, I. The state of innovation in drug development. *Clin. Pharmacol. Ther.* **83**, 227-230, (2008).

9. DiMasi, J.A., Feldman, L., Seckler, A. & Wilson, A. Trends in risks associated with new drug development: Success rates for investigational drugs. *Clin. Pharmacol. Ther.* **87**, 272-277, (2009).

10. DiMasi, J.A., Hansen, R.W. & Grabowski, H.G. The price of innovation: New estimates of drug development costs. *J. Health Econ.* **22**, 151-185, (2003).

11. Mullard, A. 2011 in reflection. *Nat. Rev. Drug Discov.* **11**, 6-8, (2012).

12. Li, J.W.H. & Vederas, J.C. Drug discovery and natural products: End of an era or an endless frontier? *Science* **325**, 161-165, (2009).

13. Newman, D.J. & Cragg, G.M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **75**, 311-335, (2012).

14. Newman, D.J. & Cragg, G.M. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* **70**, 461-477, (2007).

15. Cragg, G.M. & Newman, D.J. Natural product drug discovery in the next millennium. *Pharm. Biol.* **39**, 8-17, (2001).

16. Cragg, G.M., Newman, D.J. & Snader, K.M. Natural products in drug discovery and development. *J. Nat. Prod.* **60**, 52-60, (1997).

17. Entzeroth, M., Flotow, H. & Condron, P. Overview of high-throughput screening, in *Current protocols in pharmacology.* (ed S.J. Enna) Ch. Unit 9.4, 9.4.1-9.4.27 (John Wiley & Sons, Inc., 2009).

18. da Rocha, A.B., Lopes, R.M. & Schwartsmann, G. Natural products in anticancer therapy. *Curr. Opin. Pharmacol.* **1**, 364-369, (2001).

19. Altmann, K.-H. & Gertsch, J. Anticancer drugs from nature-natural products as a unique source of new microtubule-stabilizing agents. *Nat. Prod. Rep.* **24**, 327-357, (2007).

20. Tobinick, E.L. The value of drug repositioning in the current pharmaceutical market. *Drug News Perspect.* **22**, 119-125, (2009).

21. Ashburn, T.T. & Thor, K.B. Drug repositioning: Identifying and developing new uses for existing drugs. *Nat. Rev. Drug Discov.* **3**, 673-683, (2004).

22. Mandal, S., Moudgil, M.n. & Mandal, S.K. Rational drug design. *Eur. J. Pharmacol.* **625**, 90-100, (2009).

23. Huggins, D.J., Sherman, W. & Tidor, B. Rational approaches to improving selectivity in drug design. *J. Med. Chem.* **55**, 1424-1444, (2012).

24. Wilhelm, S., Carter, C., Lynch, M., Lowinger, T., Dumas, J., Smith, R.A., Schwartz, B., Simantov, R. & Kelley, S. Discovery and development of sorafenib: A multikinase inhibitor for treating cancer. *Nat. Rev. Drug Discov.* **5**, 835-844, (2006).

25. Mayr, L.M. & Bojanic, D. Novel trends in high-throughput screening. *Curr. Opin. Pharmacol.* **9**, 580-588, (2009).

26. Scannell, J.W., Blanckley, A., Boldon, H. & Warrington, B. Diagnosing the decline in pharmaceutical r&d efficiency. *Nat. Rev. Drug Discov.* **11**, 191-200, (2012).

27. Horrobin, D.F. Realism in drug discovery—could cassandra be right? *Nat. Biotechnol.* **19**, 1099-1100, (2001).

28. Keserü, G.M. & Makara, G.M. The influence of lead discovery strategies on the properties of drug candidates. *Nat. Rev. Drug Discov.* **8**, 203-212, (2009).

29. An, W.F. & Tolliday, N.J. Introduction: Cell-based assays for high-throughput screening., in *Methods in molecular biology.* Vol. 486 (ed P A Clemons) 1-12 (Humana Press, 2009).

30. Macarron, R. & Hertzberg, R.P. Design and implementation of high-throughput screening assays., in *High throughput screening, methods and protocols*. Vol. 565 (eds W.P. Jansen & P. Bernasconi) Ch. 1, 1-32 (Humana Press, 2009).

31. Fox, S., Farr-Jones, S., Sopchak, L., Boggs, A. & Comley, J. High-throughput screening: Searching for higher productivity. *J. Biomol. Screen.* **9**, 354-358, (2004).

32. Roses, A.D. Pharmacogenetics in drug discovery and development: A translational perspective. *Nat. Rev. Drug Discov.* **7**, 807-817, (2008).

33. Thomas, C. The impact of assay technology as applied to safety assessment in reducing compound attrition in drug discovery. *Expert Opin. Drug Discov.* **7**, 109-122, (2012).

34. Zaman, G.J.R., de Roos, J.A.D.M., Blomenröhr, M., van Koppen, C.J. & Oosterom, J. Cryopreserved cells facilitate cell-based drug discovery. *Drug Discov. Today* **12**, 521-526, (2007).

35. Astashkina, A., Mann, B. & Grainger, D.W. A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. *Pharmacol. Ther.* **134**, 82-106, (2012).

36. Justice, B.A., Badr, N.A. & Felder, R.A. 3d cell culture opens new dimensions in cell-based assays. *Drug Discov. Today* **14**, 102-107, (2009).

37. Skelin, M., Rupnik, M. & Cencič, A. Pancreatic beta cell lines and their applications in diabetes mellitus research. *ALTEX* **27**, 105-113, (2010).

38. Hirschhaeuser, F., Menne, H., Dittfeld, C., West, J., Mueller-Klieser, W. & Kunz-Schughart, L.A. Multicellular tumor spheroids: An underestimated tool is catching up again. *J. Biotechnol.* **148**, 3-15, (2010).

39. Ulrich, A.B., Schmied, B.M., Standop, J., Schneider, M.B. & Pour, P.M. Pancreatic cell lines: A review. *Pancreas* **24**, 111-120, (2002).

40. Abbott, A. Cell culture: Biology's new dimension. *Nature* **424**, 870-872, (2003).

41. Bhadriraju, K. & Chen, C.S. Engineering cellular microenvironments to improve cell-based drug testing. *Drug Discov. Today* **7**, 612-620, (2002).

42. Gidrol, X., Fouqué, B., Ghenim, L., Haguet, V., Picollet-D'hahan, N. & Schaack, B. 2d and 3d cell microarrays in pharmacology. *Curr. Opin. Pharmacol.* **9**, 664-668, (2009).

43. Griffith, L.G. & Swartz, M.A. Capturing complex 3d tissue physiology in vitro. *Nat. Rev. Mol. Cell Biol.* **7**, 211-224, (2006).

44. Kunz-Schughart, L.A. The use of 3-d cultures for high-throughput screening: The multicellular spheroid model. *J. Biomol. Screen.* **9**, 273-285, (2004).

45. Rimann, M. & Graf-Hausner, U. Synthetic 3d multicellular systems for drug development. *Curr. Opin. Biotechnol.* **23**, 1-7, (2012).

46. Pampaloni, F., Reynaud, E.G. & Stelzer, E.H.K. The third dimension bridges the gap between cell culture and live tissue. *Nat. Rev. Mol. Cell Biol.* **8**, 839-845, (2007).

47. Maltman, D.J. & Przyborski, S.A. Developments in three-dimensional cell culture technology aimed at improving the accuracy of in vitro analyses. *Biochem. Soc. Trans.* **38**, 1072-1075, (2010).

48. Kunz-Schughart, L.A., Kreutz, M. & Knuechel, R. Multicellular spheroids: A three-dimensional in vitro culture system to study tumour biology. *Int. J. Exp. Pathol.* **79**, 1-23, (1998).

49. Nirmalanandhan, V., Duren, A., Hendricks, P., Vielhauer, G. & Sittampalam, G. Activity of anticancer agents in a three-dimensional cell culture model. *Assay Drug Dev. Technol.* **8**, 581-590, (2010).

50. Bates, R.C., Edwards, N.S. & Yates, J.D. Spheroids and cell survival. *Crit. Rev. Oncol. Hematol.* **36**, 61-74, (2000).

51. Pickl, M. & Ries, C.H. Comparison of 3d and 2d tumor models reveals enhanced her2 activation in 3d associated with an increased response to trastuzumab. *Oncogene* **28**, 461-468, (2008).

52. Centers for Disease Control and Prevention. National diabetes fact sheet: National estimates and general information on diabetes and prediabetes in the united states, 2011. (U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta, GA, 2011).

53. Boyle, J.P., Thompson, T.J., Gregg, E.W., Barker, L.E. & Williamson, D.F. Projection of the year 2050 burden of diabetes in the us adult population:

Dynamic modeling of incidence, mortality, and prediabetes prevalence. *Population Health Metrics* **8**, 1-12, (2010).

54. International Diabetes Federation. *One adult in ten will have diabetes by 2030, press release*, <http://www.idf.org/media-events/press-releases/2011/diabetes-atlas-5th-edition> (2011).

55. American Diabetes Association. Economic costs of diabetes in the u.S. In 2007. *Diabetes Care* **31**, 596-615, (2007).

56. Huang, H.-H., Novikova, L., Williams, S.J., Smirnova, I.V. & Stehno-Bittel, L. Low insulin content of large islet population is present in situ and in isolated islets. *Islets* **3**, 6-13, (2011).

57. Transparency Market Research. *Global diabetes devices & drugs market (2011- 2016)*, <http://www.transparencymarketresearch.com/diabetes-devices-market.html> (2011).

58. Modi, P. Diabetes beyond insulin: Review of new drugs for treatment of diabetes mellitus. *Curr. Drug Discov. Technol.* **4**, 39-47, (2007).

59. Bolen, S., Feldman, L., Vassy, J., Wilson, L., Yeh, H.C., Marinopoulos, S., Wiley, C., Selvin, E., Wilson, R. & Bass, E.B. Systematic review: Comparative effectiveness and safety of oral medications for type 2 diabetes mellitus. *Ann. Intern. Med.* **147**, 386-399, (2007).

60. IDF Clinical Guidelines Task Force. Global guidelines for type 2 diabetes. 1-80, (International Diabetes Federation, Brussels, 2005).

61. IDF Clinical Guidelines Task Force. Global guideline for type 2 diabetes: Recommendations for standard, comprehensive, and minimal care. *Diabet. Med.* **23**, 579-593, (2006).

62. McGuire, D.K. & Inzucchi, S.E. New drugs for the treatment of diabetes mellitus: Part i: Thiazolidinediones and their evolving cardiovascular implications. *Circulation* **117**, 440-449, (2008).

63. Grundy, S.M. Advancing drug therapy of the metabolic syndrome. *Nat. Rev. Drug Discov.* **8**, 341-341, (2009).

64. Ravassard, P., Hazhouz, Y., Pechberty, S., Bricout-Neveu, E., Armanet, M., Czernichow, P. & Scharfmann, R. A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. *J. Clin. Immunol.* **121**, 3589-3597, (2011).

65. Weir, G.C. & Bonner-Weir, S. Finally! A human pancreatic β cell line. *J. Clin. Immunol.* **121**, 3395-3397, (2011).

66. Montanya, E. Islet- and stem-cell-based tissue engineering in diabetes. *Curr. Opin. Biotechnol.* **15**, 435-440, (2004).

67. Hopcroft, D.W., Mason, D.R. & Scott, R.S. Structure-function relationships in pancreatic islets: Support for intraislet modulation of insulin secretion. *Endocrinology* **117**, 2073-2080, (1985).

68. Unger, R.H. & Orci, L. Paracrinology of islets and the paracrinopathy of diabetes. *Proceedings of the National Academy of Sciences* **107**, 16009-16012, (2010).

69. Weir, G.C., Halban, P.A., Meda, P., Wollheim, C.B., Orci, L. & Renold, A.E. Dispersed adult rat pancreatic islet cells in culture: A, b, and d cell function. *Metabolism.* **33**, 447-453, (1984).

70. Bavamian, S., Klee, P., Britan, A., Populaire, C., Caille, D., Cancela, J., Charollais, A. & Meda, P. Islet-cell-to-cell communication as basis for normal insulin secretion. *Diabetes Obes. Metab.* **9**, 118-132, (2007).

71. Kelly, C., Parke, H.G., McCluskey, J.T., Flatt, P.R. & McClenaghan, N.H. The role of glucagon- and somatostatin-secreting cells in the regulation of insulin release and beta-cell function in heterotypic pseudoislets. *Diabetes Metab. Res. Rev.* **26**, 525-533, (2010).

72. Kelly, C., McClenaghan, N.H. & Flatt, P.R. Role of islet structure and cellular interactions in the control of insulin secretion. *Islets* **3**, 41-47, (2011).

73. Kelly, C., Guo, H., McCluskey, J., Flatt, P. & McClenaghan, N. Comparison of insulin release from min6 pseudoislets and pancreatic islets of langerhans reveals importance of homotypic cell interactions. *Pancreas* **00**, 1-8, (2010).

74. Halban, P.A., Wollheim, C.B., Blondel, B., Meda, P., Niesor, E.N. & Mintz, D.H. The possible importance of contact between pancreatic islet cells for the control of insulin release. *Endocrinology* **111**, 86-94, (1982).

75. Pipeleers, D., Veld, P.I., Maes, E. & Winkel, M.V.D. Glucose-induced insulin release depends on functional cooperation between islet cells. *Proc. Natl. Acad. Sci. U. S. A.* **79**, 7322-7325, (1982).

76. Samols, E., Bonner-Weir, S. & Weir, G.C. Intra-islet insulin-glucagon-somatostatin relationships. *Clin. Endocrinol. Metab.* **15**, 33-58, (1986).

77. Kayali, A.G., Flores, L.E., Lopez, A.D., Kutlu, B., Baetge, E., Kitamura, R., Hao, E., Beattie, G.M. & Hayek, A. Limited capacity of human adult islets expanded in vitro to redifferentiate into insulin-producing beta-cells. *Diabetes* **56**, 703-708, (2007).

78. Efrat, S. Regulation of insulin secretion: Insights from engineered β-cell lines. *Ann. N. Y. Acad. Sci.* **1014**, 88-96, (2004).

79. Wang, H., Zhang, W., Cai, H., Xu, S., Sui, W., Jiang, Y., Deng, S. & Lou, J. A-cell loss from islet impairs its insulin secretion in vitro and in vivo. *Islets* **3**, 58-65, (2011).

80. MacGregor, R.R., Williams, S.J., Tong, P.Y., Kover, K., Moore, W.V. & Stehno-Bittel, L. Small rat islets are superior to large islets in in vitro function and in transplantation outcomes. *Am. J. Physiol. Endocrinol. Metab.* **290**, E771-E779, (2006).

81. Nam, K.-H., Yong, W., Harvat, T., Adewola, A., Wang, S., Oberholzer, J. & Eddington, D.T. Size-based separation and collection of mouse pancreatic islets for functional analysis. *Biomed Microdevices* **12**, 865-874, (2010).

82. Williams, S.J., Huang, H.-H., Kover, K., Moore, W., Berkland, C., Singh, M., Smirnova, I.V., MacGregor, R.R. & Stehno-Bittel, L. Reduction of diffusion barriers in isolated rat islets improves survival, but not insulin secretion or transplantation outcome. *Organogenesis* **6**, 115-124, (2010).

83. Su, Z., Xia, J., Shao, W., Cui, Y., Tai, S., Ekberg, H., Corbascio, M., Chen, J. & Qi, Z. Small islets are essential for successful intraportal transplantation in a diabetes mouse model. *Scand. J. Immunol.* **72**, 504-510, (2010).

84. Lehmann, R., Zuellig, R., Kugelmeier, P., Baenninger, P., Moritz, W., Perren, A., Clavien, P., Weber, M. & Spinas, G. Superiority of small islets in human islet transplantation. *Diabetes* **56**, 594-603, (2007).

85. World Health Organization. *Diabetes: Fact sheet n°312*, <http://www.who.int/mediacentre/factsheets/fs312/en/> (2011).

86. Lefèbvre, P. Diabetes as a paracrinopathy of the islets of langerhans. *Eur. Endocrinol.* **7**, 79-83, (2011).

87. Brissova, M., Shostak, A., Shiota, M., Wiebe, P.O., Poffenberger, G., Kantz, J., Chen, Z., Carr, C., Jerome, W.G., Chen, J., Baldwin, H.S., Nicholson, W., Bader, D.M., Jetton, T., Gannon, M. & Powers, A.C. Pancreatic islet production of vascular endothelial growth factor-a is essential for islet vascularization, revascularization, and function. *Diabetes* **55**, 2974-2985, (2006).

88. Halban, P., Powers, S., George, K. & Bonner-Weir, S. Spontaneous reassociation of dispersed adult rat pancreatic islet cells into aggregates with three-dimensional architecture typical of native islets. *Diabetes* **36**, 783-790, (1987).

89. Hopcroft, D.W., Mason, D.R. & Scott, R.S. Insulin secretion from perifused rat pancreatic pseudoislets. *In Vitro Cell. Dev. Biol.* **21**, 421-427, (1985).

90. Kodama, S., Kojima, K., Furuta, S., Chambers, M., Paz, A. & Vacanti, C. Engineering functional islets from cultured cells. *Tissue Eng. Part A* **15**, 3321-3329, (2009).

91. Lee, D., Park, S., Nam, J. & Byun, Y. Optimal aggregation of dissociated islet cells for functional islet-like cluster. *J. Biomater. Sci. Polym. Ed.* **19**, 441-452, (2008).

92. Matta, S.G., Wobken, J.D., Williams, F.G. & Bauer, G.E. Pancreatic islet cell reaggregation systems: Efficiency of cell reassociation and endocrine cell topography of rat islet-like aggregates. *Pancreas* **9**, 439-449, (1994).

93. O'Sullivan, E., Johnson, A., Omer, A., Hollister-Lock, J., Bonner-Weir, S., Colton, C. & Weir, G. Rat islet cell aggregates are superior to islets for transplantation in microcapsules. *Diabetologia* **53**, 937-945, (2010).

94. Kelm, J., Ehler, E., Nielsen, L., Schlatter, S., Perriard, J. & Fussenegger, M. Design of artificial myocardial microtissues. *Tissue Eng.* **10**, 201-214, (2004).

95. Kelm, J. & Fussenegger, M. Microscale tissue engineering using gravity-enforced cell assembly. *Trends Biotechnol.* **22**, 195-202, (2004).

96. Kelm, J.M., Diaz Sanchez-Bustamante, C., Ehler, E., Hoerstrup, S.P., Djonov, V., Ittner, L. & Fussenegger, M. Vegf profiling and angiogenesis in human microtissues. *J. Biotechnol.* **118**, 213-229, (2005).

97. Cavallari, G., Zuellig, R., Lehmann, R., Weber, M. & Moritz, W. Rat pancreatic islet size standardization by the "hanging drop" technique. *Transplant. Proc.* **39**, 2018-2020, (2007).

98. Lin, R.-Z., Chou, L.-F., Chien, C.-C.M. & Chang, H.-Y. Dynamic analysis of hepatoma spheroid formation: Roles of e-cadherin and β1-integrin. *Cell Tissue Res.* **324**, 411-422, (2006).

99. Hsiao, A.Y., Tung, Y.-C., Kuo, C.-H., Mosadegh, B., Bedenis, R., Pienta, K.J. & Takayama, S. Micro-ring structures stabilize microdroplets to enable long term spheroid culture in 384 hanging drop array plates. *Biomed Microdevices*, 1-12, (2011).

100. Hsiao, A.Y., Tung, Y.-C., Qu, X., Patel, L.R., Pienta, K.J. & Takayama, S. 384 hanging drop arrays give excellent z-factors and allow versatile formation of co-culture spheroids. *Biotechnol. Bioeng.*, 1-11, (2011).

101. Tung, Y.-C., Hsiao, A.Y., Allen, S.G., Torisawa, Y.-s., Ho, M. & Takayama, S. High-throughput 3d spheroid culture and drug testing using a 384 hanging drop array. *The Analyst* **136**, 473-478, (2011).

102. Battersby, B.J. & Trau, M. Novel miniaturized systems in high-throughput screening. *Trends Biotechnol.* **20**, 167-173, (2002).

103. Wölcke, J. & Ullmann, D. Miniaturized hts technologies - uhts. *Drug Discov. Today* **6**, 637-646, (2001).

104. Williams, S.J., Wang, Q., MacGregor, R.R., Siahaan, T., Stehno-Bittel, L. & Berkland, C. Adhesion of pancreatic beta cells to biopolymer films. *Biopolymers* **91**, 676-685, (2009).

105. Huang, H.-H., Ramachandran, K. & Stehno-Bittel, L. A replacement for islet equivalents with improved reliability and validity. *Acta Diabetol.*, (2012).

106. Mata, A., Fleischman, A. & Roy, S. Characterization of polydimethylsiloxane (pdms) properties for biomedical micro/nanosystems. *Biomed Microdevices* **7**, 281-293, (2005).

107. Bu, M., Melvin, T., Ensell, G. & Wilkinson, J. A new masking technology for deep glass etching and its microfluidic application. *Sensors and Actuators A* **115**, 476-482, (2004).

108. Iliescu, C., Tay, F.E.H. & Miao, J. Strategies in deep wet etching of pyrex glass. *Sensors and Actuators A: Physical* **133**, 395-400, (2007).

109. Bretzel, R.G., Jahr, H., Eckhard, M., Martin, I., Winter, D. & Brendel, M.D. Islet cell transplantation today. *Langenbecks Arch* **392**, 239-253, (2007).

110. Digon, B.J. History of islet transplantation. *Curr. Diab. Rep.* **9**, 312-316, (2009).

111. Ichii, H. & Ricordi, C. Current status of islet cell transplantation. *J. Hepatobiliary. Pancreat. Surg.* **16**, 101-112, (2009).

112. Korsgren, O. & Nilsson, B. Improving islet transplantation: A road map for a widespread application for the cure of persons with type i diabetes. *Curr. Opin. Organ Transplant.* **14**, 683-687, (2009).

113. Vaithilingam, V., Sundaram, G. & Tuch, B. Islet cell transplantation. *Curr. Opin. Organ Transplant.* **13**, 633-638, (2008).

114. Shapiro, A., Lakey, J., Ryan, E., Korbutt, G., Toth, E., Warnock, G., Kneteman, N. & Rajotte, R. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N. Engl. J. Med.* **343**, 230-238, (2000).

115. Landsberg, D.N. & Shapiro, J. Kidney, pancreas, and pancreatic islet transplantation. *BC Med. J.* **52**, 189-196, (2010).

116. Vardanyan, M., Parkin, E., Gruessner, C. & Rodriguez Rilo, H.L. Pancreas vs. Islet transplantation: A call on the future. *Curr. Opin. Organ Transplant.* **15**, 124-130, (2010).

117. Jansson, L. & Carlsson, P.-O. Graft vascular function after transplantation of pancreatic islets. *Diabetologia* **45**, 749-763, (2002).

118. Menger, M.D., Vajkoczy, P., Beger, C. & Messmer, K. Orientation of microvascular blood flow in pancreatic islet isografts. *J. Clin. Immunol.* **93**, 2280-2285, (1994).

119. Marks, V., Tan, K.S., Stagner, J.I. & Samols, E. Intra-islet cellular interrelationships. *Biochem. Soc. Trans.* **18**, 103-104, (1990).

120. Weber, L.M., Hayda, K.N. & Anseth, K.S. Cell-matrix interactions improve beta-cell survival and insulin secretion in three-dimensional culture. *Tissue Eng. Part A* **14**, 1959-1968, (2008).

121. Kim, J.B. Three-dimensional tissue culture models in cancer biology. *Semin. Cancer Biol.* **15**, 365-377, (2005).

122. Khademhosseini, A., Langer, R., Borenstein, J. & Vacanti, J.P. Microscale technologies for tissue engineering and biology. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 2480-2487, (2006).

123. Jia, D., Dajusta, D. & Foty, R. Tissue surface tensions guide in vitro self-assembly of rodent pancreatic islet cells. *Dev. Dyn.* **236**, 2039-2049, (2007).

124. Layer, P., Robitzki, A., Rothermel, A. & Willbold, E. Of layers and spheres: The reaggregate approach in tissue engineering. *Trends Neurosci.* **25**, 131-134, (2002).

125. Lin, R.-Z. & Chang, H.-Y. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol. J.* **3**, 1172-1184, (2008).

126. Jakab, K., Neagu, A., Mironov, V., Markwald, R. & Forgacs, G. Engineering biological structures of prescribed shape using self-assembling multicellular systems. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 2864-2869, (2004).

127. Jakab, K., Norotte, C., Marga, F., Murphy, K., Vunjak-Novakovic, G. & Forgacs, G. Tissue engineering by self-assembly and bio-printing of living cells. *Biofabrication* **2**, 1-14, (2010).

128. Napolitano, A.P., Chai, P., Dean, D.M. & Morgan, J.R. Dynamics of the self-assembly of complex cellular aggregates on micromolded nonadhesive hydrogels. *Tissue Eng.* **13**, 2087-2094, (2007).

129. Friedrich, J., Ebner, R. & Kunz-Schughart, L.A. Experimental anti-tumor therapy in 3-d: Spheroids – old hat or new challenge? *Int. J. Radiat. Biol.* **83**, 849-871, (2007).

130. Nirmalanandhan, V.S. & Sittampalam, G.S. Stem cells in drug discovery, tissue engineering, and regenerative medicine: Emerging opportunities and challenges. *J. Biomol. Screen.* **14**, 755-768, (2009).

131. National Institute of Health. *Stem cell basics*, <http://stemcells.nih.gov/info/basics/> (2009).

132. McCall, M.D., Toso, C., Baetge, E.E. & Shapiro, A.M.J. Are stem cells a cure for diabetes? *Clin. Sci.* **118**, 87-97, (2010).

133. Chandra, V., G, S., Phadnis, S., Nair, P. & Bhonde, R. Generation of pancreatic hormone-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells. *Stem Cells* **27**, 1941-1953, (2009).

134. Santamaria, X., Massasa, E.E., Feng, Y., Wolff, E. & Taylor, H.S. Derivation of insulin producing cells from human endometrial stromal stem cells and use in the treatment of murine diabetes. *Mol. Ther.* **19**, 2065-2071, (2009).

135. Wang, X. & Ye, K. Three-dimensional differentiation of embryonic stem cells into islet-like insulin-producing clusters. *Tissue Eng. Part A* **15**, 1941-1952, (2009).

136. Chen, L.-B., Jiang, X.-B. & Yang, L. Differentiation of rat marrow mesenchymal stem cells into pancreatic islet beta-cells. *World J. Gastroenterol.* **10**, 3016-3020, (2004).

137. D'Amour, K.A., Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A., Kroon, E., Carpenter, M.K. & Baetge, E.E. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nat. Biotechnol.* **24**, 1392-1401, (2006).

138. Matveyenko, A.V., Georgia, S., Bhushan, A. & Butler, P.C. Inconsistent formation and non function of insulin positive cells from pancreatic

endoderm derived from human embryonic stem cells in athymic nude rats. *Am. J. Physiol. Endocrinol. Metab.* **299**, E713-E720, (2010).

139. Sakata, N., Chan, N.-K., Chrisler, J., Obenaus, A. & Hathout, E. Bone marrow cells produce nerve growth factor and promote angiogenesis around transplanted islets. *World J. Gastroenterol.* **16**, 1215-1220, (2010).

140. Rosengren, A.H., Taneera, J., Rymo, S. & Renström, E. Bone marrow transplantation stimulates pancreatic β-cell replication after tissue damage. *Islets* **1**, 10-18, (2009).

141. Park, K.S., Kim, Y.S., Kim, J.H., Choi, B.K., Kim, S.H., Oh, S.H., Ahn, Y.R., Lee, M.S., Lee, M.K., Park, J.B., Kwon, C.H., Joh, J.W., Kim, K.W. & Kim, S.J. Influence of human allogenic bone marrow and cord blood-derived mesenchymal stem cell secreting trophic factors on atp (adenosine-5'-triphosphate)/adp (adenosine-5'-diphosphate) ratio and insulin secretory function of isolated human islets from cadaveric donor. *Transplant. Proc.* **41**, 3813-3818, (2009).

142. Jung, E.-J., Kim, S.-C., Wee, Y.-M., Kim, Y.-H., Choi, M.Y., Jeong, S.-H., Lee, J., Lim, D.-G. & Han, D.-J. Bone marrow-derived mesenchymal stromal cells support rat pancreatic islet survival and insulin secretory function in vitro. *Cytotherapy* **13**, 19-29, (2011).

143. Wu, H., Ye, Z. & Mahato, R.I. Genetically modified mesenchymal stem cells for improved islet transplantation. *Mol. Pharm.* **8**, 1458-1470, (2011).

144. Berman, D.M., Willman, M.A., Han, D., Kleiner, G., Kenyon, N.M., Cabrera, O., Karl, J.A., Wiseman, R.W., O'Connor, D.H., Bartholomew, A.M. & Kenyon, N.S. Mesenchymal stem cells enhance allogeneic islet engraftment in nonhuman primates. *Diabetes* **59**, 2558-2568, (2010).

145. Leventhal, J., Abecassis, M., Miller, J., Gallon, L., Ravindra, K., Tollerud, D.J., King, B., Elliott, M.J., Herzig, G., Herzig, R. & Ildstad, S.T. Chimerism and tolerance without gvhd or engraftment syndrome in hla-mismatched combined kidney and hematopoietic stem cell transplantation. *Sci. Transl. Med.* **4**, 1-10, (2012).

146. Kikugawa, R., Katsuta, H., Akashi, T., Yatoh, S., Weir, G.C., Sharma, A. & Bonner-Weir, S. Differentiation of copas-sorted non-endocrine pancreatic cells into insulin-positive cells in the mouse. *Diabetologia* **52**, 645-652, (2009).

147. Bi, D., Chen, F.G., Zhang, W.J., Zhou, G.D., Cui, L., Liu, W. & Cao, Y. Differentiation of human multipotent dermal fibroblasts into islet-like cell clusters. *BMC Cell Biol.* **11**, 1-7, (2010).

148. Chen, C., Chai, J., Singh, L., Kuo, C.-Y., Jin, L., Feng, T., Marzano, S., Galeni, S., Zhang, N., Iacovino, M., Qin, L., Hara, M., Stein, R., Bromberg, J.S., Kyba, M. & Ku, H.T. Characterization of an in vitrodifferentiation assay for pancreatic-like cell development from murine embryonic stem cells: Detailed gene expression analysis. *Assay Drug Dev. Technol.* **9**, 403-419, (2011).

149. Kerr-Conte, J., Vandewalle, B., Moerman, E., Lukowiak, B., Gmyr, V., Arnalsteen, L., Caiazzo, R., Sterkers, A., Hubert, T., Vantyghem, M.C. & Pattou, F. Upgrading pretransplant human islet culture technology requires human serum combined with media renewal. *Transplantation* **89**, 1154-1160, (2010).